EFFECT OF GLUCOSE AND REDUCING AGENTS ON SYNGAS FERMENTATION BY CLOSTRIDIA SPECIES P11

By

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EFFECT OF GLUCOSE AND REDUCING AGENTS

ON SYNGAS FERMENTATION BY

CLOSTRIDIA SPECIES P11

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CHAPTER 1

INTRODUCTION

The obtainable energy on earth is available from different renewable and non renewable resources. Until the late 1800s, our society was greatly dependent on plant biomass to meet its energy demand. The discovery of crude oil in the 19th century led to industrialization and a better standard of living. Diminishing oil resources along with ever increasing oil demand and environmental concerns have made it imperative to develop a low-cost, economical and energy efficient renewable fuel such as ethanol. According to Huber et al. (2006), biofuels are considered to be the only sustainable source of liquid fuels. Biofuels are more environmentally friendly than fossil fuels, as they emit less green house gases than fossil fuels and thus reduce the global warming effect (Lashof and Ahuja 1990). USDA has estimated that the net farm income in the U.S would increase from $3 billion to $6 billion if switchgrass became an energy crop, thus biofuels have a positive effect on agriculture as well (Huber et al. 2006).

Fuel grade ethanol can be produced from biomass materials. Ethanol can be used as an additive to gasoline or as an alternate transportation fuel. According to the Department of Energy (1997) a 10% blend of ethanol (E10) can reduce carbon monoxide, carbon dioxide and nitrogen oxides emissions by 25-30%, 6-10% and 20% respectively. Ethanol is considered a better additive than methyl tertiary butyl ether (MTBE) as it is biodegradable and has a higher octane number than MTBE which helps to increase the energy efficiency of the fuel. The use of ethanol also has economic advantages as it helps

in reducing the amount of imported oil and decreases the trade deficit. According to Renewable Fuels Association, use of ethanol reduced the U.S trade deficit by $5.1 billion and supported the creation of 147,000 jobs in 2004 (Anonymous 2005).

Bio-ethanol (ethanol derived from renewable sources) is produced by the conversion of carbon- based feedstock. Some of the current methods of producing ethanol from biomass are: a) direct fermentation of sugar, b) acid/enzymatic hydrolysis of biomass followed by fermentation and c) gasification of biomass followed by fermentation using a biological catalyst.

The gasification/fermentation method is a new method in which the biomass is partially oxidized in a gasifier to yield synthesis gas or syngas (a mixture of carbon monoxide, carbon dioxide and hydrogen) followed by the fermentation of these gases by microbial catalysts such as *Clostridium carboxidivorans*, *C. ljungdahlii* or P11, to yield ethanol and other products. Further development of this method is required because microbial catalysts produce ethanol at a slow rate. It is hypothesized that the overall slow rate of conversion of syngas to ethanol could be due to longer lag period or low cell mass. Furthermore, the efficiency of the process could be improved if the metabolism could be regulated by promoting the enzymes involved towards solventogenesis.

This research focuses on the microbial catalyst P11. This organism is capable of converting syngas into ethanol however, it was found to have a long lag period and low ethanol yield. The present research is based on the hypothesis that the use of passaged cells (subcultured cells) along with the use of glucose as an initial substrate could help in reducing the lag period. Yet another focus of this study is to increase the ethanol yield by

promoting the metabolism towards ethanol production, using various reducing agents such as benzyl viologen, methyl viologen and neutral red.

CHAPTER 2

OBJECTIVES

The research objectives were:

1. To study the effect of glucose as an initial substrate on the growth rate and ethanol yield of P11, due to the observed lag period and low ethanol yield.
2. To regulate the metabolism towards solventogenesis by the addition of artificial electron carriers / reducing agents.

CHAPTER 3

LITERATURE REVIEW

3.1 INTRODUCTION:

Depleting oil and gas resources along with soaring demand has increased the interest in other feedstocks for production of fuels and chemicals. High oil prices in the last few years have further increased the significance of finding an alternate fuel that is cheaper, efficient and eco-friendly. Improved refining technologies and the addition of synthetic fuels or ethanol are currently used to produce cleaner burning fuel (Henstra et al. 2007). Figure 3.1 illustrates the bioconversion of organic materials by anaerobes. The use of alternative, renewable fuels, such as ethanol has gained momentum as they can be produced from feedstocks such as waste materials (urban wastes), forest products (wood, logging residues, trees, shrubs), energy crops (corn, sorghum, grasses) or aquatic biomass (algae, water weed, water hyacinth) (Huber et al. 2006).

Biofuels hold the key for the sustainable production of liquid transportation fuels without compromising the needs of future generations. Biomass can be converted into liquid fuels by three methods (Huber et al. 2006).

- Hydrolysis/ Fermentation.
- Pyrolysis/ Fermentation.
- Gasification/ Fermentation.

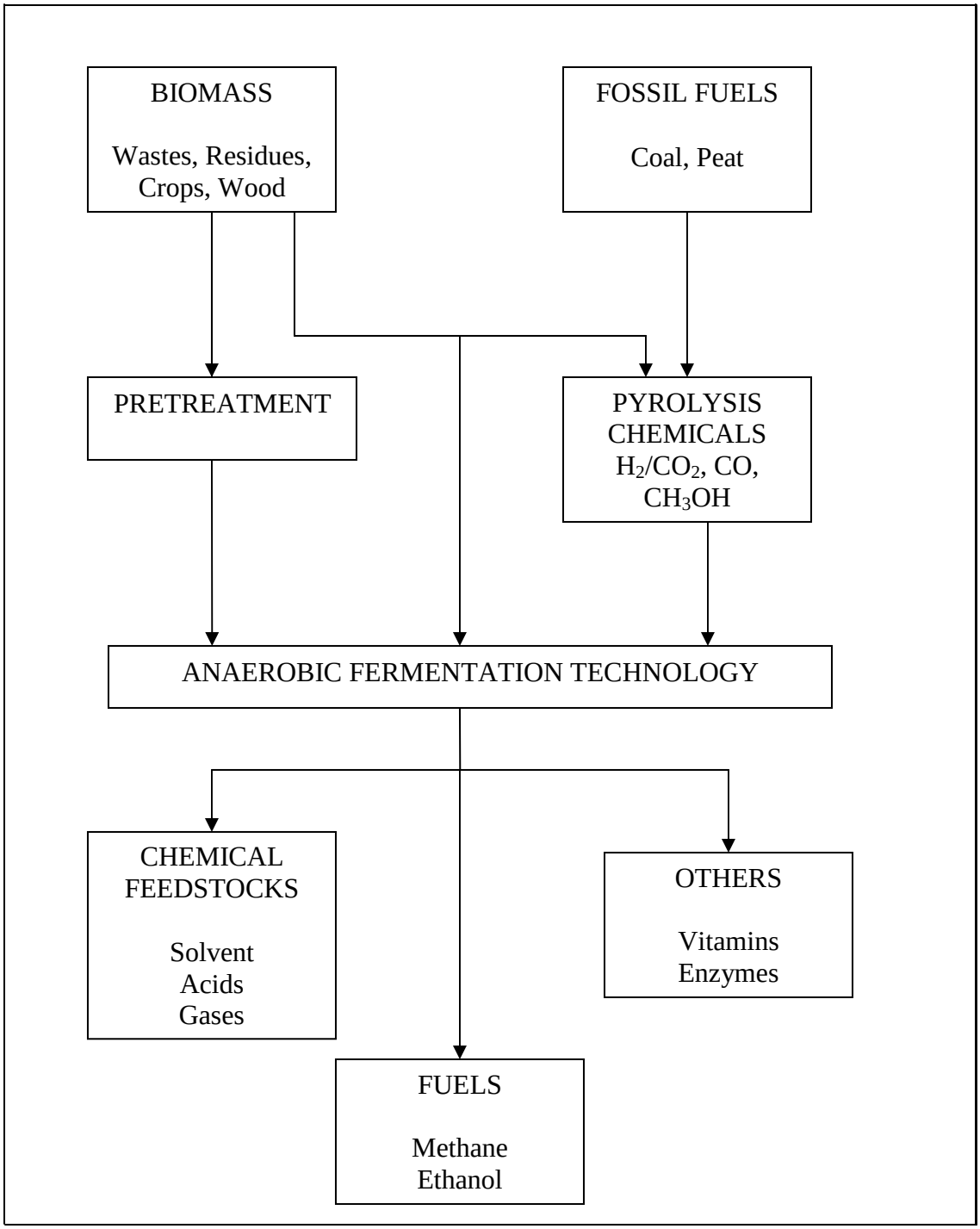

Figure 3.1: Bioconversion of organics by anaerobic bacteria (Adapted from Zeikus 1980).

Ethanol can be produced from biomass by the following methods (Clausen and Gaddy 1996):

a) Conversion of biomass to sugars by acid/enzymatic hydrolysis, followed by fermentation.

b) Conversion of biomass to syngas by gasification, followed by either catalytic conversion or fermentation.

3.2 HYDROLYSIS:

The major chemical components of the various biomass/feedstocks are cellulose, hemicellulose and lignin (Clausen and Gaddy 1996). During hydrolysis, the polysaccharides cellulose and hemicellulose are hydrolyzed to glucose and a mixture of 5- and 6- carbon sugars respectively, by using a weak acid (sulfuric acid, hydrochloric acid) solution or enzymes (cellulase). The hydrolysis conversion of cellulose to glucose molecules is shown in Equation 3.1. Glucose is then converted to ethanol by fermentation (Huber et al. 2006) as shown in Equation 3.2.

$$(C_6H_{10}O_5)_n + nH_2O \rightarrow nC_6H_{12}O_6 \qquad (3.1)$$

$$nC_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \qquad (3.2)$$

This process has several operational problems. It suffers from conversion inefficiencies due to the lignin fraction of the biomass which cannot be converted to ethanol and also pentose sugars produced from hemicellulose cannot be fermented to ethanol with proven technology (Clausen and Gaddy 1996). Moreover, the efficiency of this process depends on the nature of the substrate. The substrate must be clean, well milled and pretreated to increase the yield, however, milling operations and pretreatment methods of the substrate are expensive (Cheremisinoff et al. 1980; Lin and Tanaka 2006).

3.3 GASIFICATION:

Gasification is the process in which carbonaceous materials react with air/oxygen/steam to produce a gas product called synthesis gas that contains CO, CO_2, H_2 and N_2 in various proportions (Huber et al. 2006). The technology of gasification is applicable for any carbonaceous material, which includes biomass materials such as switchgrass, sugar beets, sweet sorghum, etc. Gasification of these materials converts the carbon structure of lignin, cellulose and hemicellulose to simple synthesis gas components. These components serve as substrates for microbial metabolism yielding products such as ethanol, butanol, and acetic acid (Phillips et al. 1994**).** Biomass gasification is accomplished through the following set of reactions (Rao 2005):

Oxidation of Carbon:

$$C + ½\ O_2 \rightarrow CO \qquad (3.3)$$

$$C + O_2 \rightarrow CO_2 \qquad (3.4)$$

Boudouard Reaction:

$$C + CO_2 \rightarrow 2\ CO \qquad (3.5)$$

Water-gas shift reaction:

$$CO + H_2O \rightarrow CO_2 + H_2 \qquad (3.6)$$

Methane Formation:

$$C + 2\ H_2 \rightarrow CH_4 \qquad (3.7)$$

Reverse Methanation Reaction:

$$CH_4 + H_2O \rightarrow CO + 3\ H_2 \qquad (3.8)$$

The overall stochiometry for the formation of ethanol and acetate from syngas components (CO and CO_2/H_2) are as follows (Klasson et al. 1992):

$$6\ CO + 3\ H_2O \rightarrow C_2H_5OH + 4\ CO_2 \quad (3.9)$$

$$2\ CO_2 + 6\ H_2 \rightarrow C_2H_5OH + 3\ H_2O \quad (3.10)$$

$$4\ CO + 2\ H_2O \rightarrow CH_3COOH + 2\ CO_2 \quad (3.11)$$

$$2\ CO_2 + 4\ H_2 \rightarrow CH_3COOH + 2\ H_2O \quad (3.12)$$

Syngas is a major building block in the production of fuels and chemicals and it can be manufactured from natural gas, coal, petroleum, biomass and organic wastes. The importance of syngas chemistry came into light in the early 20^{th} century as syngas can be used to produce a large number of complex chemicals with the use of both heterogeneous and homogenous catalysts. The direct use of syngas as a clean fuel for integrated gasification combined cycle (IGCC) units for the generation of electricity. Fuel ethanol, obtained from syngas fermentation, is considered an area of potential growth (Wender 1996). The present syntheses of major chemicals from syngas are shown in Figure 3.2. Syngas fermentation offers several advantages over the other means of production of biofuels such as greater resistance to biocatalysts, lower energy costs and independence of a fixed H_2: CO ratio. A further advantage of syngas fermentation over the other conventional methods, is the higher specificity of the microbial catalysts (Heiskanen et al. 2007; Henstra et al. 2007). Moreover, the nearly irreversible nature of biological reactions allows complete conversion which increases the overall efficiency of the process (Younesi et al. 2005).

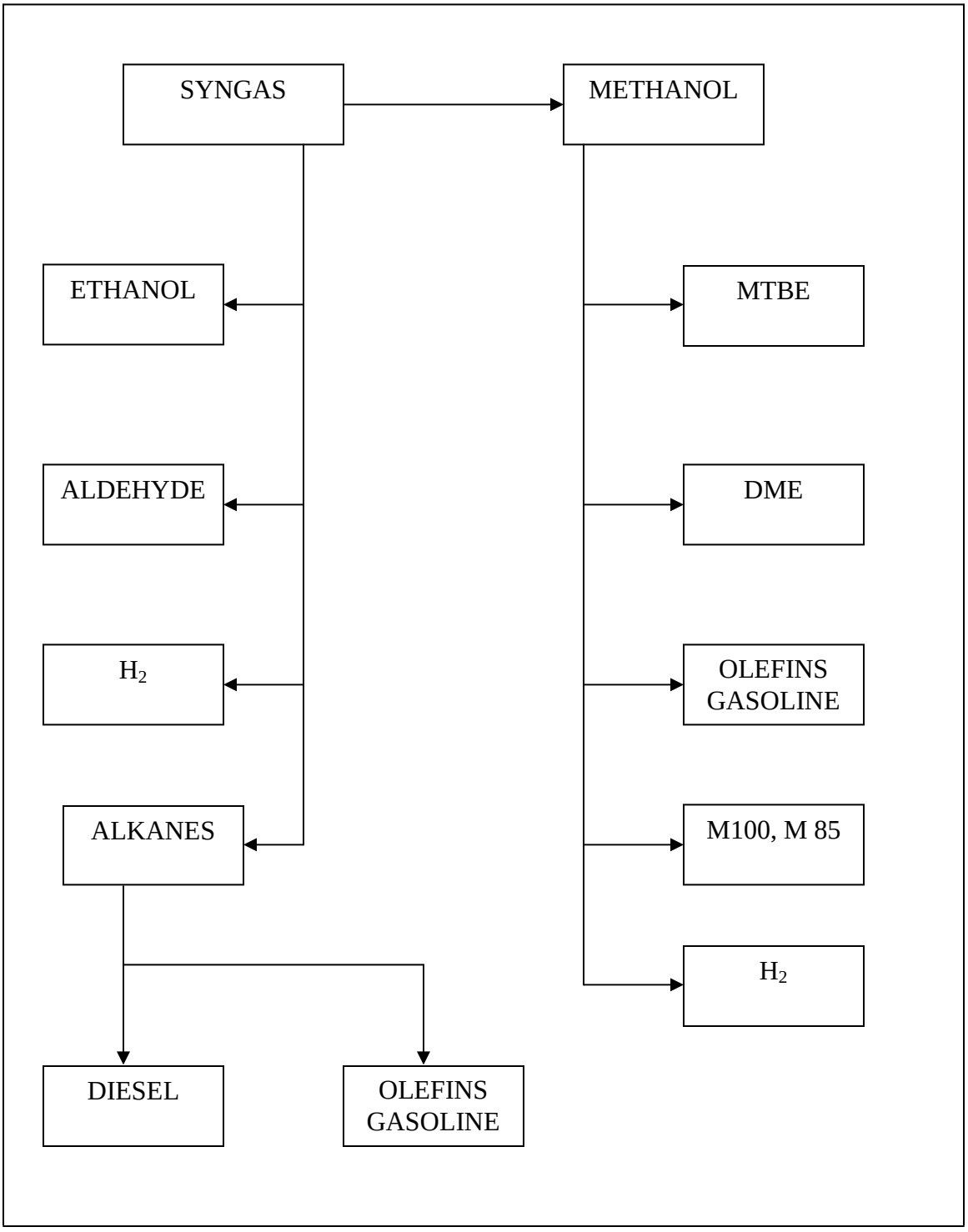

Figure.3.2: Pathways for fuel production from syngas (Adapted from Huber et al. 2006)

MTBE- Methyl tert-butyl ether, DME – Dimethyl ether.

3.4 ACETOGENS:

Acetogenic bacteria are obligate anaerobes that convert simple inorganic substrates such as CO, H_2 and CO_2 to acetic acid. These organisms use the acetyl-CoA pathway, also known as the "Wood/Ljungdahl" pathway for the synthesis of acetyl-CoA, conservation of energy and growth (Drake 1992). Acetogens derive energy for growth by reducing carbon dioxide as the terminal electron acceptor to acetyl-CoA through the acetogenic pathway proposed by Ljungdahl and Wood (Phillips et al. 1994). Acetogenic bacteria, such as *Acetobacterium woodii* (Klasson et al. 1991)**,** *Butyribacterium methylotrophicum (*Heiskanen et al. 2007; Lynd et al. 1982), *Clostridium ljundahlii* (Klasson et al. 1991; Younesi et al. 2005), *Clostridium thermoaceticum*, *Peptostrptococcus productus* (Vega et al. 1989) and *Clostridium carboxidivorans* (Rajagopalan et al. 2002) use syngas components as a source of carbon and energy, and produce acetate, ethanol and carbon dioxide.

3.5 ACETYL-CoA PATHWAY:

In the acetyl-CoA pathway, two molecules of carbon dioxide are reduced to acetyl-CoA in a process that involves eight reducing equivalents/electrons, as shown in equation (3.13). The carbon dioxide molecules can be reduced to form either a methyl group by the action of formate dehydrogenase or a carbonyl group by acetyl-CoA synthase/carbon monoxide dehydrogenase (CODH). Acetyl-CoA is then converted to yield acetate via phosphotransacetylase and acetate (Drake 1995; Hu et al. 1982; Winzer et al. 1997).

CO_2 + [8 reducing equivalents] ----> Acetyl- CoA----> Acetate + Biomass. (3.13)

In the case of syngas fermentation, the gas supplies the organism with carbon monoxide, carbon dioxide and hydrogen. The enzyme carbon monoxide dehydrogenase oxidizes carbon monoxide into carbon dioxide, thus providing the carbon dioxide molecules that are required (Ljungdahl 1986).

$$CO + H_2O \leftrightarrow CO_2 + 2\ H^+ + 2\ e^- \quad (3.14)$$

The acetyl-CoA pathway can be considered to have two reaction paths, both of which converge in the formation of acetyl-CoA by the enzyme acetyl-CoA synthase. In the methyl branch, six reducing equivalents are consumed in the overall reaction of carbon dioxide to the methyl group of acetyl-CoA, while the carbonyl branch consumes two reducing equivalents (Drake 1992). Figure 3.3 shows the two reaction paths of the pathway.

Acetyl-CoA is considered as an important intermediate in the Wood-Ljungdahl pathway as it acts as a precursor for lipids, amino acids, nucleotides and carbohydrates (Ljungdahl 1986). To produce cellular materials, acetyl-CoA goes through anabolism in which it is converted into phosphoenolpyruvate, which is a major intermediate in the conversion to cellular material. For the conservation of energy, acetyl-CoA also undergoes catabolism in which it is converted to acetate while producing adenosine triphosphate (ATP). This branch of the pathway is known as the acidogenic phase and is favored by the bacterium as it results in the production of ATP (Ahmed 2006). Moreover, this path also results in the lowering of pH owing to the production of acid and is also marked by the rapid growth of organisms. This phase is then followed by a second, slower growth phase with solvent formation, that is known as the solventogenic phase (Rao and Mutharasan 1987). During the solventogenic phase, the organism utilizes the reducing potential of NADH

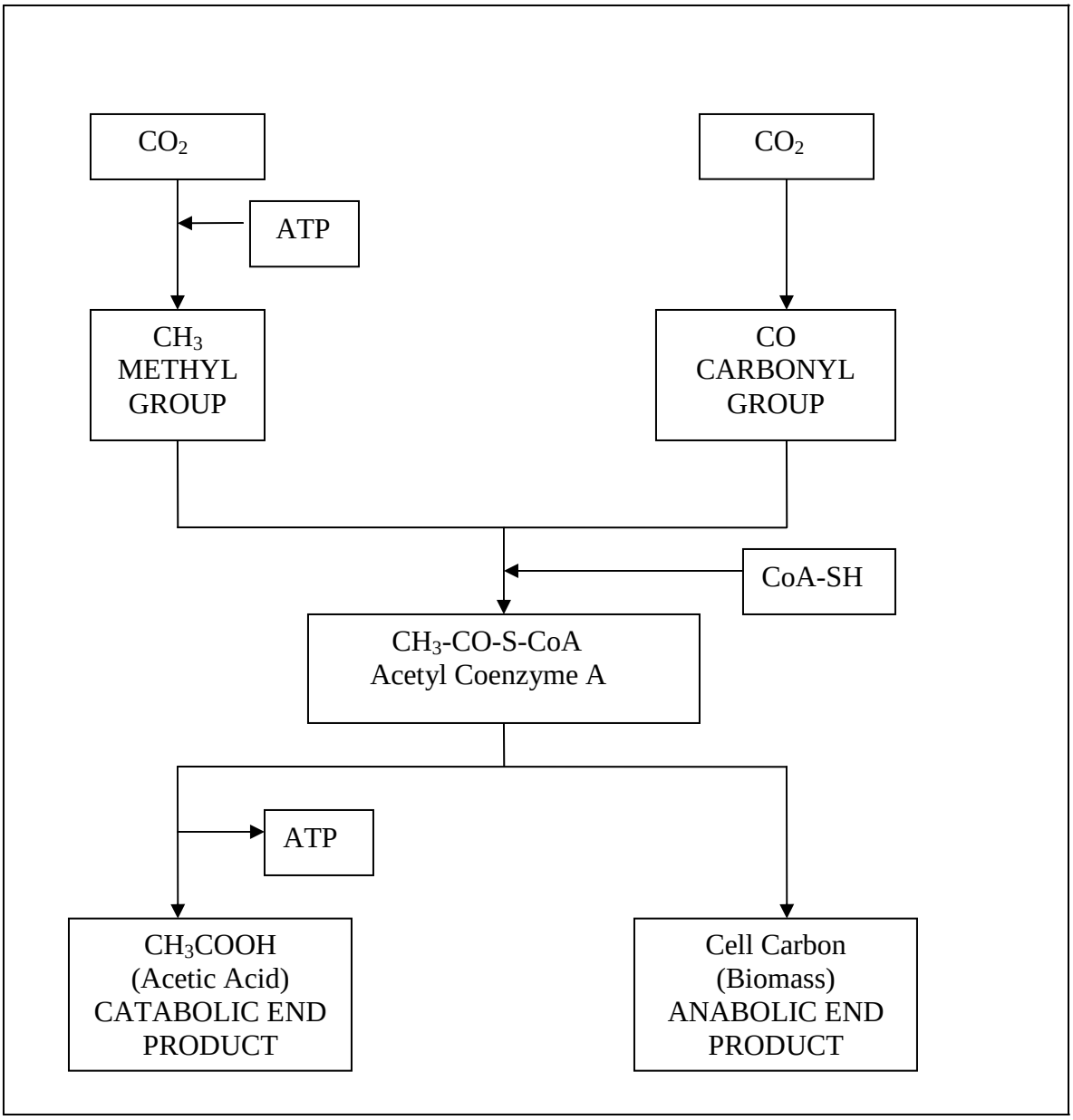

Figure 3.3: Overview of acetyl-CoA pathway (Adapted from Drake 1992).The Methyl and the Carbonyl branches of the pathway are shown.

[reduced form of nicotinamide adenine dinucleotide (NAD+)] to produce acetaldehyde, which then is converted to ethanol by the action of alcohol dehydrogenase as shown in Figure 3.4 and by equation 3.15 and 3.16. CoA-SH denotes coenzyme –A with a sulfhydryl functional group.

$$\text{Acetyl-CoA} + \text{NADH} + \text{H}^+ \rightarrow \text{Acetaldehyde} + \text{NAD}^+ + \text{CoA-SH} \quad (3.15)$$

$$\text{Acetaldehyde} + \text{NADH} + \text{H+} \rightarrow \text{Ethanol} + \text{NAD}^+ \quad (3.16)$$

Many acetogens are also known to produce butanol and butyric acid from acetyl Co-A through acetoacetyl-CoA (Rao and Mutharasan 1987). Various factors are involved in triggering the metabolic shift from the acidogenic phase to the solventogenic phase. These include pH, ATP demand, availability of nutrients, availability of reducing equivalents, enzyme activities and electron flow (Ahmed 2006; Vasconcelos et al. 1994). It has been observed that yeast extract has an effect on the product ratio, resulting in higher ethanol production by *C. ljundahlii*. When yeast extract concentration was reduced, an increase in solvent concentration was observed, leading to the conclusion that ethanol production is not related to growth (Klasson et al. 1992; Younesi et al. 2005). Enhanced solvent production was observed in *C. acetobutylicum* when ATP demand was low or when ATP generation was of high efficiency. In the same study, it was shown that an increase in the availability of reducing energy also leads to an increase in solvent production (Girbal et al. 1995).

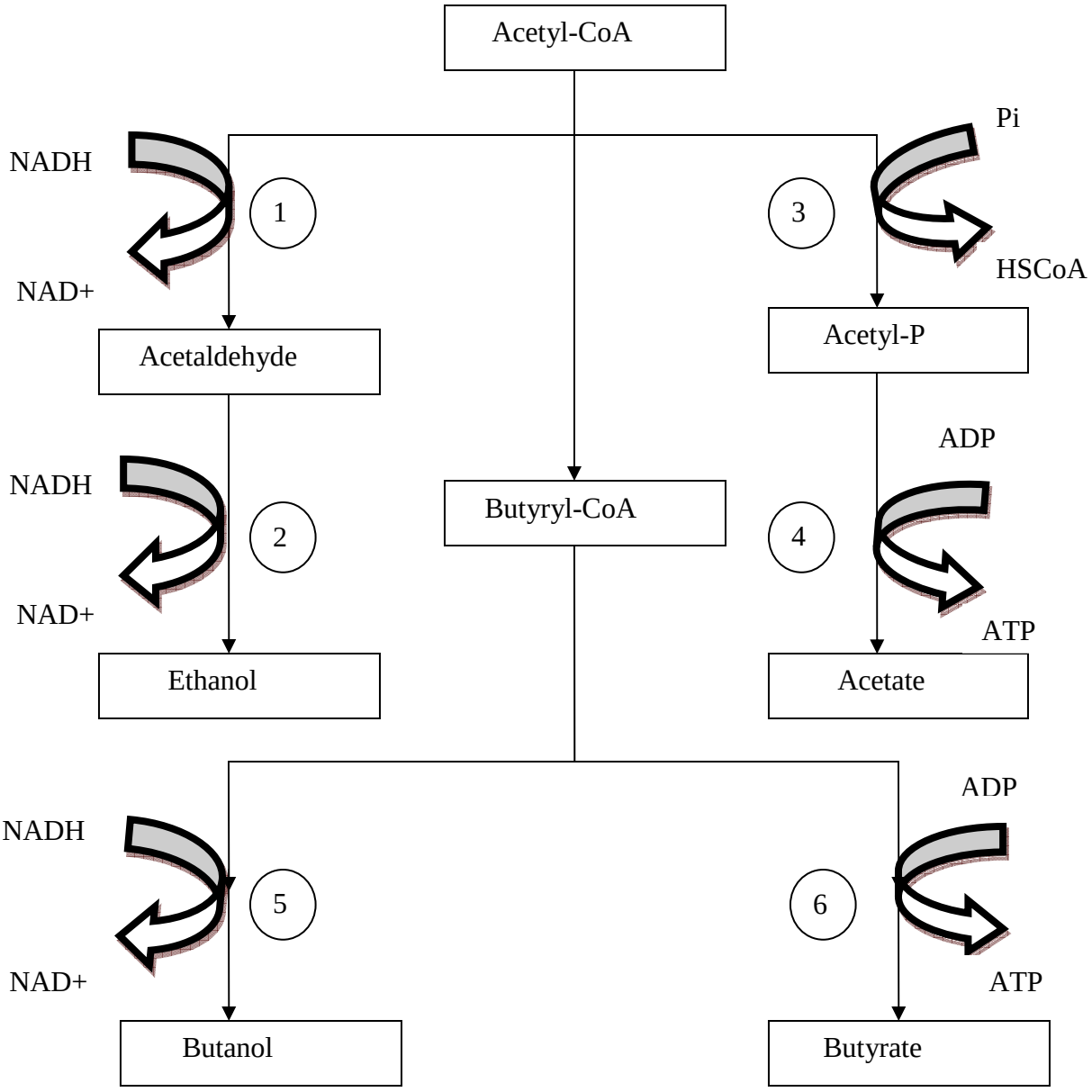

Figure 3.4:Role of Acetyl-CoA as an intermediate in *C.acetobutylicum* (Adapted from Vasconcelos et al. 1994) 1- Acetaldehyde dehydrogenase, 2- Alcohol dehydrogenase, 3- Phosphotransacetylase, 4-Acetate kinase, 5- Butanol dehydrogenase and 6- Butyrate kinase.

3.6 ENERGETICS/ELECTRON FLOW OF ACETYL-COA PATHWAY:

For acetogens that grow autotrophically, it's observed that one ATP molecule is used in activating formate in the formyl-THF synthetase reaction as shown in Figure 3.5. Under standard conditions, the reduction of carbon dioxide to produce acetic acid is exergonic as it produces an ATP molecule. As the ATP consumed during formate activation is recovered in the later steps from acetyl-CoA, there is no net gain of ATP. Thus, the generation of additional ATP is postulated to occur by electron transport phosphorylation that is coupled to the reduction of CO_2 to acetate (Fuchs 1986). Studies on the membranes of the organisms *C. thermoautotrophicum* and *C. thermoaceticum* has shown that the membranes of these homoacetogens contain the enzymes hydrogenase, NADH-ferredoxin, oxidoreductase, a pyrroloquinoline (PQQ)-dependent methanol dehydrogenase, carbon monoxide dehydrogenase (CODH), methylene-tetra hydrofolate reductase and a proton dependent ATPase. Several electron carriers such as cytochrome (cyt) b560 and cyt b556, ferredoxin, rubedoxin, menaquinone, 2-methyl-3-heptaprenyl-1,4-naphtoquinone (MK-7) were also isolated from the membranes (Fuchs 1986; Ljungdahl 1994; Wood and Ljungdahl 1991). Furthermore, the presence of ion-porters (sodium and proton) along with ATPases has further supported the concept of electron transport in CO_2 metabolism involving a chemiosmotic mechanism/electron transport mediated phosphorylation as they are capable of coupling the membrane mediated electron transport to the generation of proton motive force (Diekert 1990; Drake 1992; Ljungdahl 1994). Such chemiosmotic mechanism has been observed in other organisms, like *C. hydrogenoformans*, which is a hydrogenogenic carboxydotroph - meaning it conserve metabolic energy through the formation of hydrogen. In this organism, CO

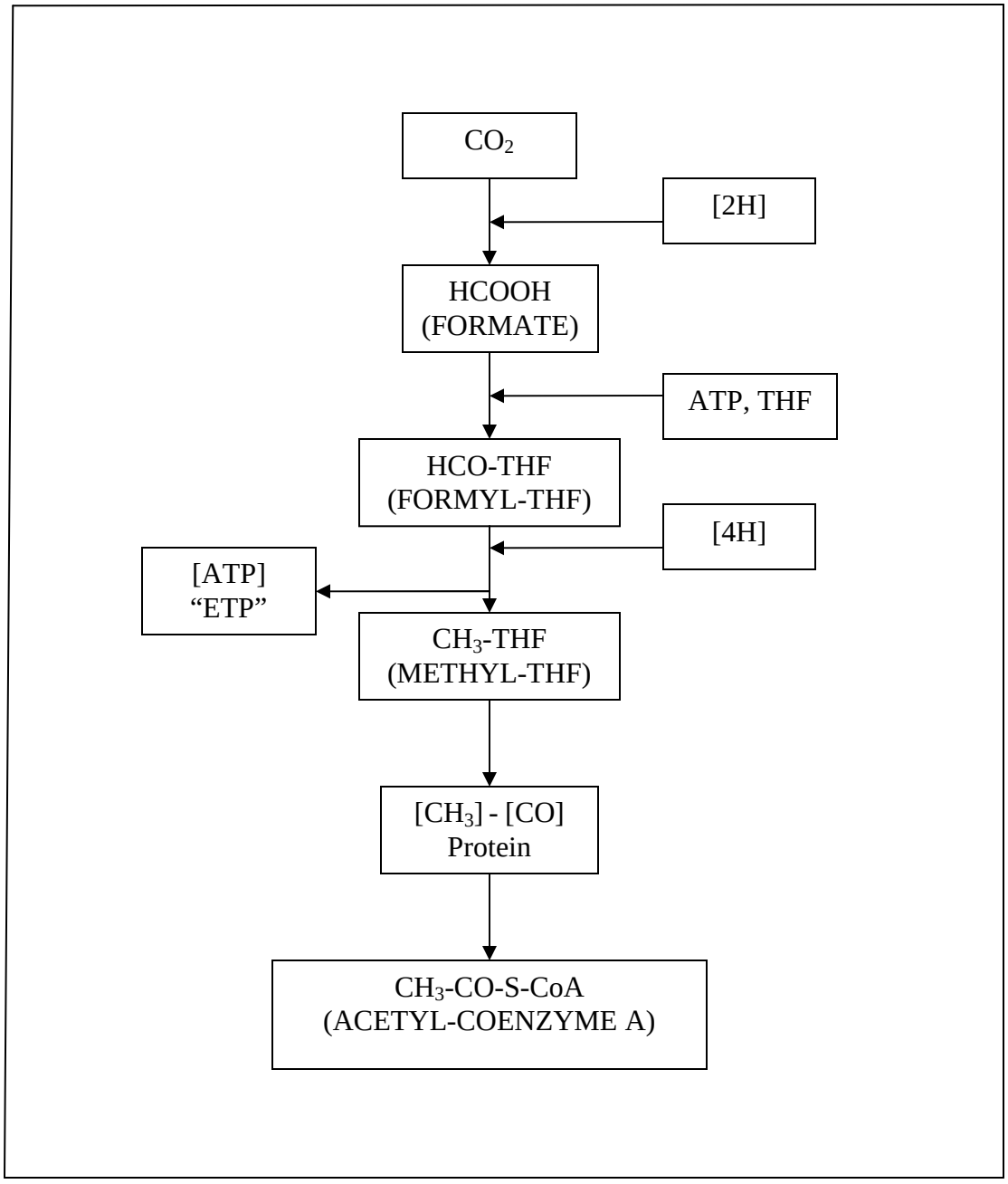

Figure 3.5: Carbonyl branch of the Acetyl-CoA pathway. Bracketed ATP indicates the conservation of energy via chemiosmotic processes and electron transport phosphorylation (ETP) (Adapted from Ljungdahl 1994).

serves as the carbon source, electron donor and energy source. The oxidation of CO to CO_2 is coupled to ATP formation indirectly and provides the required reducing equivalent. This helps the organism to balance between ATP generation and formation of reducing equivalents required for its growth (Henstra et al. 2007). Figures 3.5 and 3.6 portray the proposed chemiosmotic mechanism in acetogens.

3.7 EFFECT OF NUTRIENTS:

Bacterial growth in batch culture has four phases: a) lag phase, b) exponential phase, c) stationary phase, and d) death phase. During lag phase the cells adapt themselves in order to acclimate to the environment before the onset of the exponential phase (Alcamo 1983; Swinnen et al. 2004). A greater understanding of the factors affecting this phase is required as an increase in the lag phase limits the efficiency of alcohol production from fermentation. Various factors such as environmental conditions (pH, temperature, nutrients etc.), phenotype of the bacteria, the growth stage of cells and inoculum size are known to influence the length of the lag phase (Swinnen et al. 2004) .

Experiments with *Hydrogenomonas eutropha* demonstrated that the organism always had a lag period of 1-4 hr when an inoculum from the post-exponential phase was used; however, this lag period was reduced when the inoculum was taken from the exponential phase. Moreover, the lag period of this organism was also found to be affected by the partial pressure of carbon dioxide and oxygen. Decreasing the partial pressure to 0.05 atm reduced the lag period, indicating that either excess carbon dioxide, oxygen or both were significant factors for the observed lag period (Repaske et al. 1971).

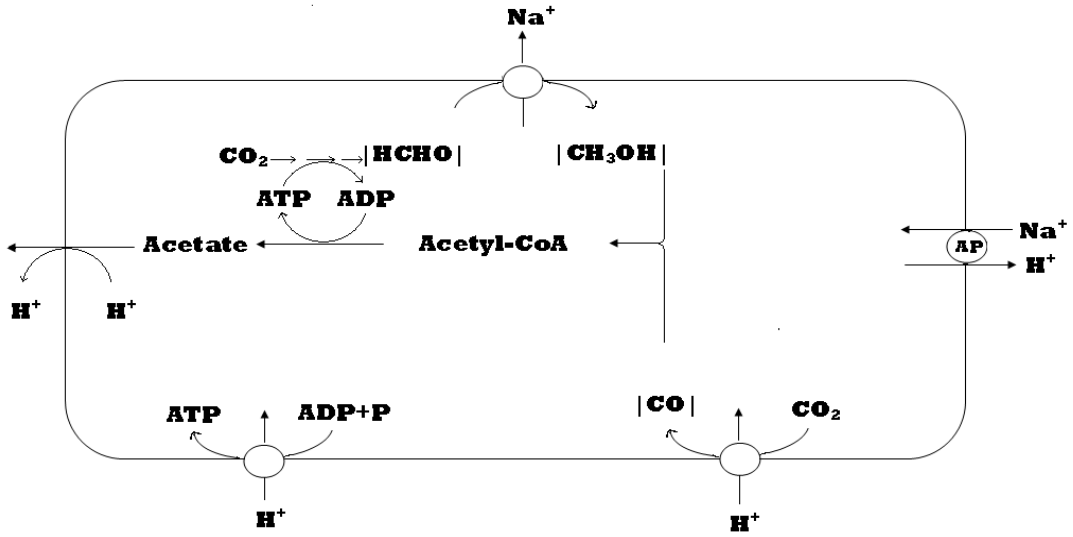

Figure 3.6: Scheme of energy conservation in homoacetogenic bacteria (Adapted from Diekert 1990).

The addition of yeast extract reduced the lag period that was observed in *Lactobacillus rhamnosus* when it was grown on a medium containing hydrolyzed rice bran, which served as the nutrient source (Gao et al. 2008). Similar effects of reduced lag period were observed when the concentrations of sodium ions were reduced during the growth of the marine bacterium *Deleya aesta*. Supplementation of the medium with potassium bicarbonate or yeast extract reduced the lag period. It was concluded that a combination of transportable carbon sources reduced the lag period and increased the rate of exponential growth. It was also observed that amino acids and vitamins are capable of having similar effects on the lag period and growth rate (Berthelet and MacLeod 1989). Research on *C. acetobutylicum* has shown that when grown in a synthetic medium in the presence of a sugar, higher yield of alcohol was produced. The presence of glucose increased both the growth rate and the amount of alcohol produced (Bahl et al. 1986). *C. carboxidivorans*, an organism that is capable of growing autotrophically on syngas components as well as chemoorganotrophically on sugars, was observed to have higher growth rate when grown on fructose (Liou et al. 2005).

Vasconcelos et al. (1994) showed that the growth of *C. acetobutylicum* on a medium having glucose along with glycerol led to production of alcohol, whereas, with glucose alone, the organism produced more acid and less alcohol. Most of the present studies on syngas fermentation by anaerobes take place in a glucose limiting medium, thus glucose can be looked upon as a promising nutrient that may reduce the lag period and help in increasing the growth rate of acetogens. Switching syngas fermenting organisms grown in a glucose medium to syngas, may allow these organisms to attain higher initial growth along with increased alcohol production.

3.8 GLUCOSE METABOLISM:

Fermentation of sugars to ethanol is the best established process for the conversion of biomass to energy (Classen et al. 1999). Glucose, a monosaccharide, is an important carbohydrate that acts as a source of energy and a metabolic intermediate in living cells. The use of glucose may be either aerobic or anaerobic. Various organisms are known to use glucose anaerobically to produce ethanol. *Clostridia* are saccharolytic and are capable of using a wide range of carbohydrates. The biochemistry of the process and its technological features are well characterized. During glucose fermentation, glucose is transported into the cells and enters the glycolytic pathway (Embden- Mayerhof pathway) to produce pyruvate, ATP (energy) and NADH (reducing equivalents). The fate of pyruvate is then controlled by the cellular redox balance and the kinetic properties of the available catalytic capacities (Temudo et al. 2007). Figure 3.7 shows the possible products that can be produced by the anaerobic fermentation of glucose by a mixed culture.

C. thermoaceticum, one of the earliest acetogens to be found, is capable of converting sugars such as glucose and xylose to acetate as shown by equations (3.17) and (3.18).

$$C_6H_{12}O_6 \rightarrow 3\ CH_3COOH \qquad (3.17)$$

$$2\ C_5H_{10}O_5 \rightarrow 5\ CH_3COOH \qquad (3.18)$$

During the conversion of glucose to acetate, glucose undergoes fermentation via the Embden-Meyerhof-Parnas glycolytic pathway to yield pyruvate, which is further

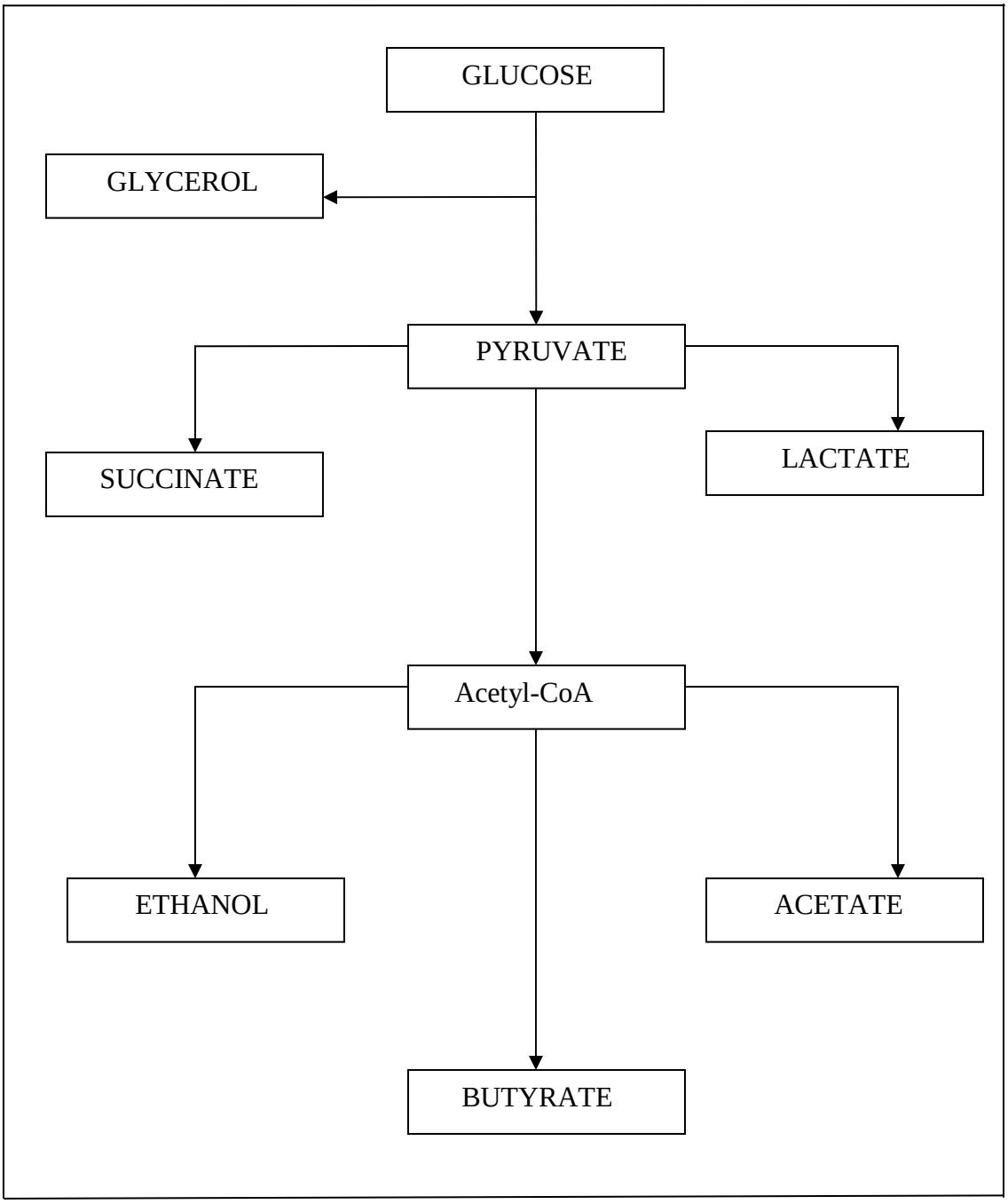

FIGURE 3.7: Glucose fermentation during anaerobic fermentation by mixed cultures (Adapted from Temudo et al. 2007).

metabolized to acetate and carbon dioxide. The carbon dioxide evolved during glucose fermentation serves as an electron acceptor and is reduced to acetate through the acetyl-CoA pathway (Ljungdahl 1986). Equations 3.19 and 3.20 show the conversion of glucose to acetate and the reduction of the carbon dioxide produced during that process to acetate.

$$C_6H_{12}O_6 + 2\ H_2O \rightarrow 2\ CH_3COOH + 2\ CO_2 + 8\ H^+ + 8\ e^- \quad (3.19)$$

$$2\ CO_2 + 8\ H^+ + 8\ e^- \rightarrow CH_3COOH + 2\ H_2O \quad (3.20)$$

Thus the homoacetic acid fermentation has a yield of 3 mol acetic acid per mol of metabolized glucose as shown by equation 3.17. The homoacetogenic fermentation involves several electron carriers such as ferredoxin and cytochrome. In *C. thermoaceticum*, the process involves tetrahydrofolate derivatives that act as an one-carbon carrier and are involved in the formation of acetate through carbon dioxide, the electron flow associated with the process is not completely understood. However, these bacteria obtain ATP by both substrate level as well as electron transport mediated phosphorylation (Zeikus 1980). Figure 3.8 shows the homoacetic fermentation pathway for glucose.

3.9 EFFECT OF ARTIFICIAL ELECTRON CARRIERS:

Artificial electron carriers are known to perform natural electron transfers by altering the distribution of $NADH/NAD^+$ ratio and thus modify metabolism by controlling the direction of carbon flow towards the solventogenic phase in *Clostridia* (Hipolito et al. 2008). Neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) was the first artificial electron carrier found to have such effect by Hongo (1958). Later,

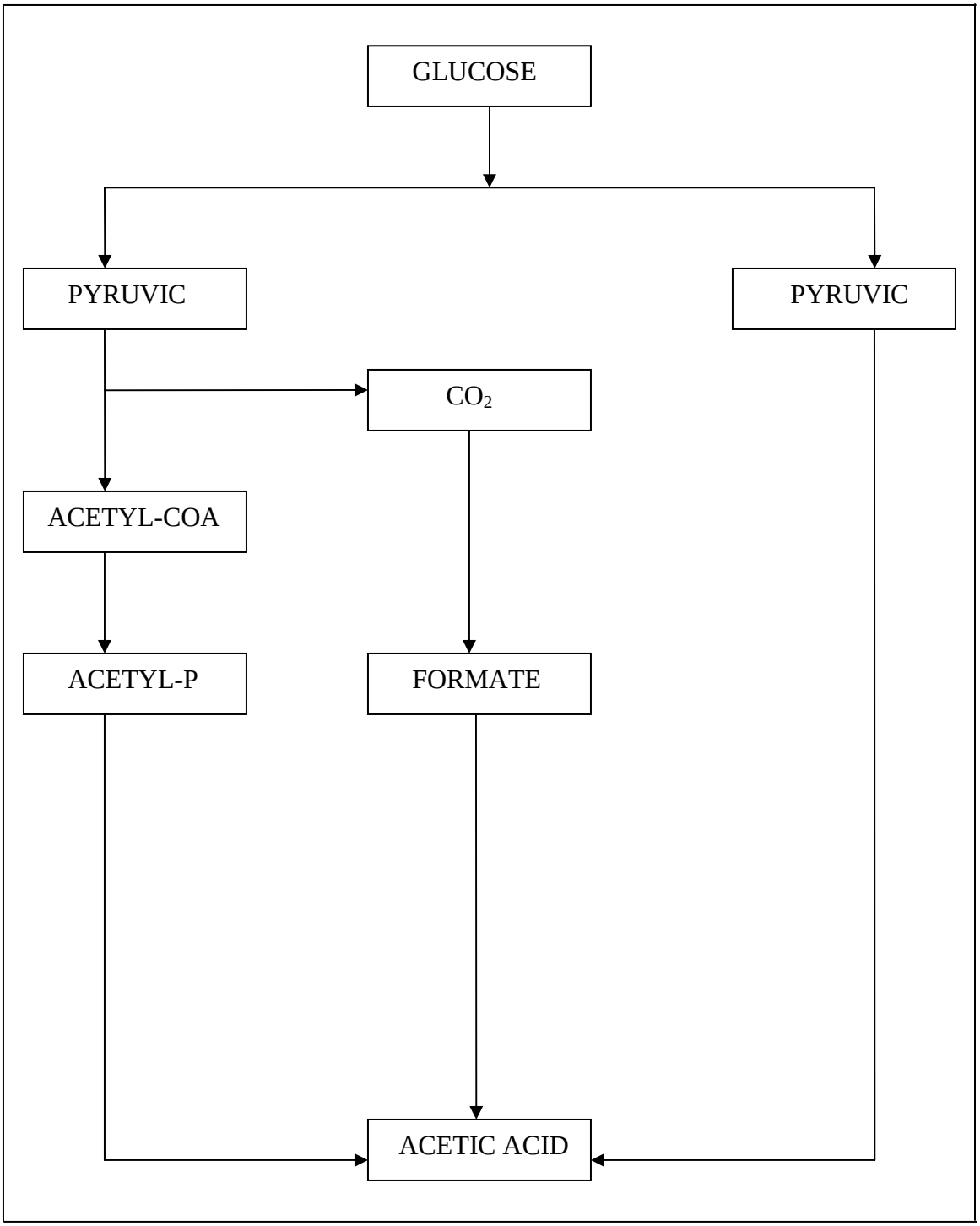

Figure 3.8: Homoacetic fermentation pathway for glucose (Adapted from Zeikus 1980).

methylene blue and viologen dyes were also found to regulate the electron flow leading to the promotion of the solventogenic phase (Girbal et al. 1995; Peguin et al. 1994). Addition of methyl viologen alters the electron flow in favor of alcohols. Methyl viologen decreases hydrogen evolution and directs electrons towards NAD, which results in increased availability of NADH, thus promoting the NADH dependent reduction of acetyl-CoA to ethanol (Rao and Mutharasan 1986). In the experiments conducted by Rao and Mutharasan (1986), a seven- fold increase in ethanol production was observed in the presence of methyl viologen with *C. acetobutylicum*. Experiments conducted on *C. acetobutylicum*, to observe the physiological events that occur during the phase shift from acidogenesis to solventogenesis, showed that the addition of methyl viologen increased NAD(P)H concentration as well as that of ATP. However, when the solventogenic phase is induced by the addition of butyrate and acetate, the intracellular ATP concentration reached a minimum before the onset of alcohol production. This leads to the assumption that shift in phase is caused by the generation of two different signals, one by the changed ATP level and the other by the NAD(P)H level (Grupe and Gottschlk 1992).

The addition of 1 mM neutral red to *C. acetobutylicum*, an organism that produces both ethanol and butanol in its solventogenic phase, resulted in the increase of ethanol by a factor of three and induced butanol formation, while a 6 mM addition of neutral red increased butanol production by 10-15%. It was also observed that the total acid produced decreased by a factor of two. At the enzyme level, the presence of neutral red increased the activity of both ethanol and butanol dehyrogenases, the enzymes that produce the respective alcohols, by seven and fourteen fold, while at the same time an

eight fold decrease in the activity of the enzyme that produces acetate, phosphotransacetylase, led to a decrease in the acetate concentration by ten fold (Girbal et al. 1995).

In another study where methyl viologen was used as an electron carrier, the addition of 1 mM methyl viologen at pH 4.5 reduced the growth rate of *C. acetobutylicum* that was grown in a media containing glucose (Peguin et al. 1994). However, the organism continued to consume sugar and produce solvent. The metabolism was inhibited by the high concentration of butanol. Methyl viologen induced the formation of butanol, lactate and glycerol in this organism and decreased the concentration of acetone, butyrate and hydrogen at the same time. It was also observed that the high concentration of butanol in the presence of methyl viologen led to the inhibition of the total metabolism. High butanol concentration has various adverse effects such as inhibition of glucose uptake, lowering intracellular level of ATP and destruction of the transmembrane pH gradient (Peguin et al. 1994).

3.10 ALCOHOL DEHYDROGENASE:

Alcohol dehydrogenase plays a significant role in the acetyl-CoA pathway. This enzyme converts acetaldehyde produced from acetyl-CoA during the solventogenesis phase to ethanol as shown by equation 3.14. Girbal et al. (1995) demonstrated in their study that the shift in the solventogenic phase is regulated by the enzymes involved in the various steps of the metabolism. They observed that the presence of artificial electron carriers increased the activities of aldehyde dehydrogenase and alcohol dehydrogenase with NADH as a cofactor. Thus, a measure of the activity of ADH could suggest the onset of solventogenesis.

CHAPTER 4

EFFECT OF GLUCOSE ON SYNGAS FERMENTATION BY P11

4.1 INTRODUCTION:

Production of ethanol from biomass through the gasification-fermentation process is considered as a good alternative to fossil fuels due to its potential low cost and availability of biomass (Ahmed et al. 2006). This process involves the conversion of biomass to syngas (a mixture of CO, CO_2, H_2 and N_2), which is then converted to ethanol by the use of microbial catalysts. It has been found that certain anaerobic bacteria are capable of using syngas components as their substrate using the acetyl-CoA pathway, also known as the Wood-Ljungdahl pathway, to produce acetic acid and ethanol (Ahmed et al. 2006; Drake 1992; Phillips et al. 1994). During syngas fermentation, acetyl-CoA is produced which acts as an important intermediate. It undergoes anabolism to provide cellular material or catabolism to provide the cells with ATP during which acid is produced, resulting in the lowering of pH. This acid- producing phase, known as acidogenesis, is then followed by a second slow growth phase, during which the organism makes use of the present reducing potential to produce alcohol. Various factors such as pH, ATP demand, electron flow, enzyme activities and availability of nutrients are known to affect this switch between the acidogenesis and solvent producing phase, also known as solventogenesis (Ahmed 2006; Vasconcelos et al. 1994). The

efficiency of alcohol production from fermentation is also affected by the lag phase of the organisms. During lag phase the cells adapt themselves in order to acclimate to the environment before the onset of the exponential phase (Alcamo 1983). An increase in the lag period limits the efficiency of alcohol production. Factors such as nutrients, pH, temperature and even inoculum size are known to influence the lag phase (Swinnen et al. 2004). Since *Clostridia* are saccharolytic and are capable of using a wide range of carbohydrates, the use of glucose as an initial substrate may increase cell concentration and alcohol production. Moreover, a previous study on *C. acetobutylicum* showed that when grown in a synthetic medium containing sugar, a higher yield of alcohol was produced compared to the culture that was grown in the absence of sugar. The presence of glucose increased both the growth rate and alcohol yield (Bahl et al. 1986). During the conversion of glucose to acetate, glucose undergoes fermentation via the Embden-Meyerhof-Parnas glycolytic pathway to yield pyruvate, which is further metabolized to acetate and carbon dioxide. The carbon dioxide evolved during glucose fermentation serves as an electron acceptor and gets reduced to acetate through the acetyl-CoA pathway (Ljungdahl 1986; Temudo et al. 2007).

The objective of the chapter is to report the effect of glucose as an initial substrate on the growth rate and ethanol yield of P11. It is hypothesized that using glucose prior to syngas will reduce lag period and increase the cell concentration which will lead to the increased production of ethanol.

4.2 MATERIALS AND METHODS:

4.2.1 Syngas:

Commercial syngas bought from Superior Specialty Gas, Inc (Tulsa, OK) was used

throughout the experiment. The composition used was 5% Hydrogen, 15% carbon dioxide, 20% carbon monoxide and 60% nitrogen. The above mentioned composition was used as it is equivalent to the composition of syngas obtained through fluidized bed gasifier using switchgrass as substrate (Datar 2003).

4.2.2 Microbial Catalyst and Culture Medium:

P11 was provided by Dr. Ralph Tanner, University of Oklahoma. This bacterium is capable of fermenting syngas (CO, CO_2, and H_2), as well as sugars, to produce alcohols and acids. The organism was grown under strict anaerobic conditions.

A defined medium containing minerals, trace metals, vitamins and reducing agents was used to prepare the inoculum and cultivate the cells. The mineral stock solution contained (per liter) 100g ammonium chloride, 4g calcium chloride, 20g magnesium sulfate, 10g potassium chloride, 10g potassium phosphate monobasic and 80g sodium chloride. The trace metal composition (per liter) was 0.2g cobalt chloride, 0.8 g ferrous ammonium sulfate, 1g manganese sulfate, 0.2g nickel chloride, 2g nitrilotriacetic acid, 0.02g sodium molybdate, 0.1g sodium selenate, 0.2g sodium tungstate and 1g zinc sulfate. The stock vitamin solution (per liter) contained 0.005g of p-amino benzoic acid, 0.002g d-biotin, 0.005g pantothenic acid, 0.002g folic acid, 0.01g MESNA, 0.005g nicotinic acid, 0.01g pyridoxine, 0.005g riboflavin, 0.005g thiamine, 0.005g thioctic acid and 0.005g vitamin B-12. The media contained (per liter) 30ml mineral stock solution,10ml trace metal solution, 10ml vitamin stock solution, 1g yeast extract, 10g morpholinoethanesulfonic acid (MES), and 10ml of 4% cysteine-sulfide solution. Resazurin solution (0.1%) was added as a redox indicator. MES was added as a biological buffer to prevent excessive

fluctuations in the pH during the course of the experiment. The initial pH of the medium was adjusted to 6 before inoculation.

4.2.3 Batch Studies:

Batch experiments were conducted in 250ml serum bottles with 100ml of liquid media to study the effect of glucose. Media composition was the same as described above for the controls; while one batch contained 0.6% (w/v) glucose and another had 1.2% (w/v) glucose. All studies were performed in triplicates. The media was boiled and purged with nitrogen for 2 minutes to remove oxygen and allowed to cool. 1ml of 4% Cysteine sulfide was added to the bottles to scavenge any remaining dissolved oxygen. Bottles were then sterilized in an autoclave (Primus Sterilizer Co. Inc, Omaha, NE) at 121ºC for 20 min. Sterilized bottles are allowed to cool. Controls were purged with syngas while the bottles containing glucose were purged with nitrogen. The bottles were then inoculated with 10ml of inoculum and placed at 37ºC on a shaker (Innova 2100, New Brunswick Scientific, Edison, NJ) at 150 rpm. Cell concentration, pH and product concentration were measured at regular intervals. While the organisms were in the exponential growth phase in the bottles containing glucose, the cells were then centrifuged at 3000 rpm for 40 min. Collected cells were then transferred to bottles containing fresh media. These bottles were then purged with nitrogen gas to 137.89 kPa. The entire procedure of cell transfer was done anaerobically in an anaerobic chamber (Coy Laboratory Products Inc., Grasslake, MI).

4.2.4 Analytical Procedures:

The optical density (OD), which is proportional to the cell concentration (includes vegetative cells, spores and dead cells), was determined using a UV-Vis

spectrophotometer (Varian Inc., Palo Alto, CA). Cell samples were collected from the bottles in 2 ml cuvettes. OD was measured at 660nm. Samples with an OD greater than 0.4 units were diluted appropriately such that the OD value obtained was within the linear range of calibration which was 0-0.4 units. A calibration chart was developed to relate OD to the dry cell weight (g/l).

Collected samples were then centrifuged at 10,000 rpm for 10 min, supernatant was collected and frozen for further analysis. Analysis was done to measure concentrations of ethanol, acetic acid and glucose using HPLC **(**Agilent 1100 series, Wilmington, DE**)** with refractive index detection. A 0.01N sulfuric acid was used as the eluent at a flow rate of 0.6ml/min with a column temperature of 60ºC.

4.2.4.1 Calibration of cell optical density (OD) against dry cell weight:

One liter of reactor broth was collected and the initial OD was measured. Dilutions were done to obtain solutions of different OD. The diluted culture was then measured for OD against water as a blank. The samples of various dilutions were then filtered by vacuum filtration with the filter paper being changed after every sample. Before filtering the sample, each of the filter papers (Whatman, Piscataway, NJ) was placed on an aluminum weighing dish (Whatman, Piscataway, NJ) and weighed. After the filtration, the filter papers were placed on the aluminum dishes and dried for a period of 24h in an oven maintained at 103ºC. The weight of the filter papers along with the aluminum dishes were weighed again and the difference in the weights were recorded. The difference in the weight correlates to the cell mass concentration (g/l). The value of the cell mass was plotted against its respective OD value. Figure 4.1 shows the relation between optical

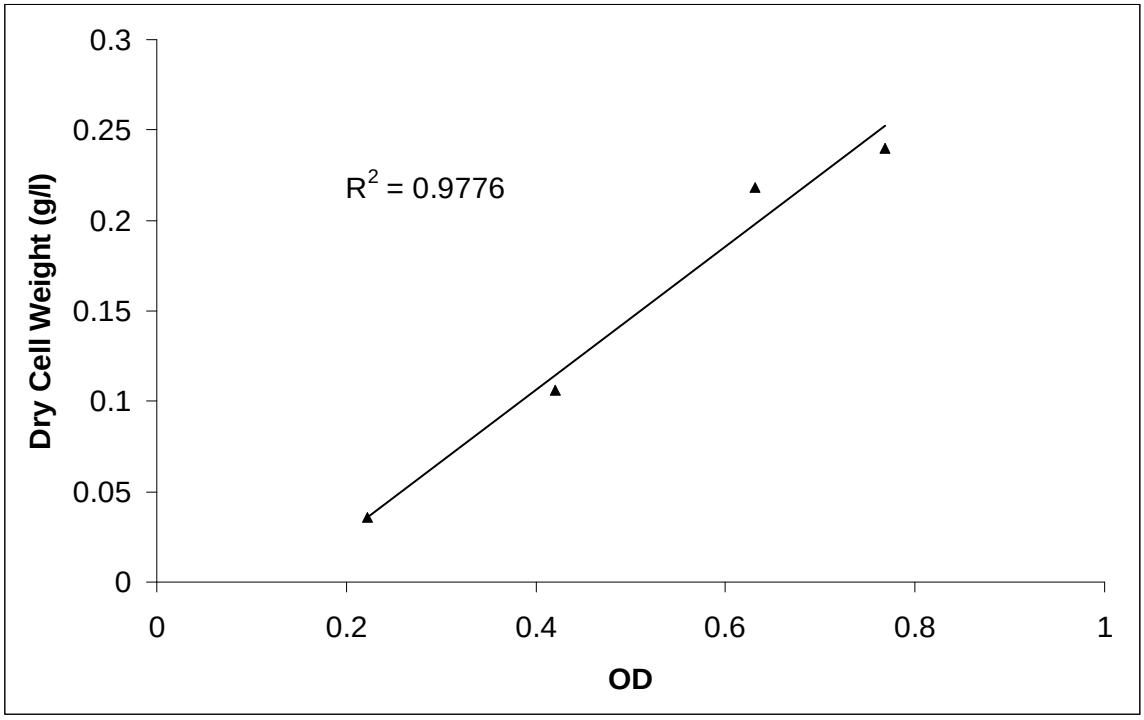

Figure 4.1: Cell optical density versus dry cell weight.

density of the cell and dry cell weight. The relationship between the dry cell weight and cell OD was observed as:

Dry Cell Weight (g/l) = 0.396* Cell OD – 0.0521

4.3 RESULTS AND DISCUSSION:

4.3.1. Cell Growth:

Figures 4.2 and 4.3 show cell growth before and after switching the cells to syngas from glucose. Growth rates were measured before the transfer of cells. Use of glucose as an initial substrate resulted in higher growth rate compared to the growth on syngas as the initial substrate of the organism (Figure 4.2).

Figures 4.4, 4.5 and 4.6 show the growth rate of the organism under the three different substrate conditions. The cells grown on glucose had a specific growth rate of 0.0466 h^{-1} (0.6% glucose) and 0.0485 h^{-1}(1.2% glucose), which were higher than the growth rate of the cells grown on syngas of 0.0327 h^{-1}. Moreover the cells initially grown on glucose continued to have a higher cell concentration throughout the experiment even after their transfer from glucose to syngas compared to the cells in the control. Furthermore, it canbe seen that the use of sub- cultured (passaged) cells along with the use of glucose as an initial substrate helped in promoting cell growth (Figure 4.2).

Figure 4.7 shows the consumption of glucose by P11 before transferring the cells to syngas. It can be seen that the cells used only an average of 1.5g/l of glucose under both conditions. The observed growth of the organism could be due to the more reduced nature of the substrate (glucose), as compared to CO and H_2, and also due to the nature of glucose to solublize in an aqueos media. With the use of syngas, there are mass transfer issues but it is not the case with glucose as it is readily soluble in the media.

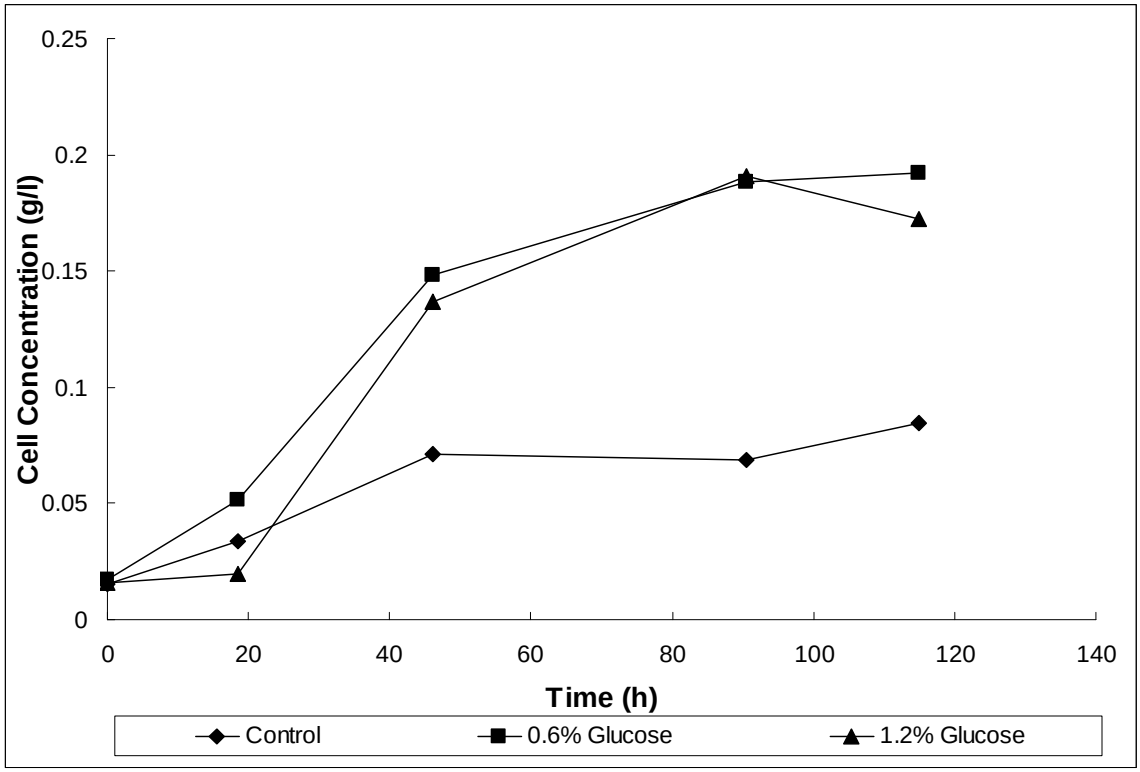

Figure 4.2: Initial cell growth on syngas (control) and glucose.

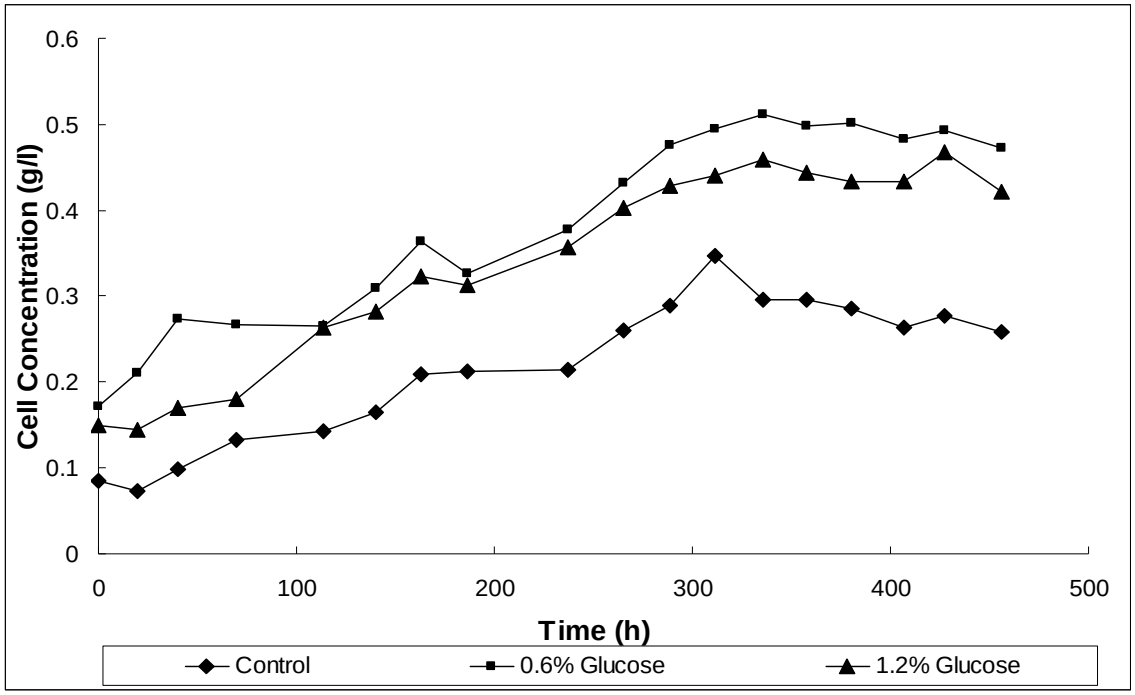

Figure 4.3: Cell growth after transferring to syngas.

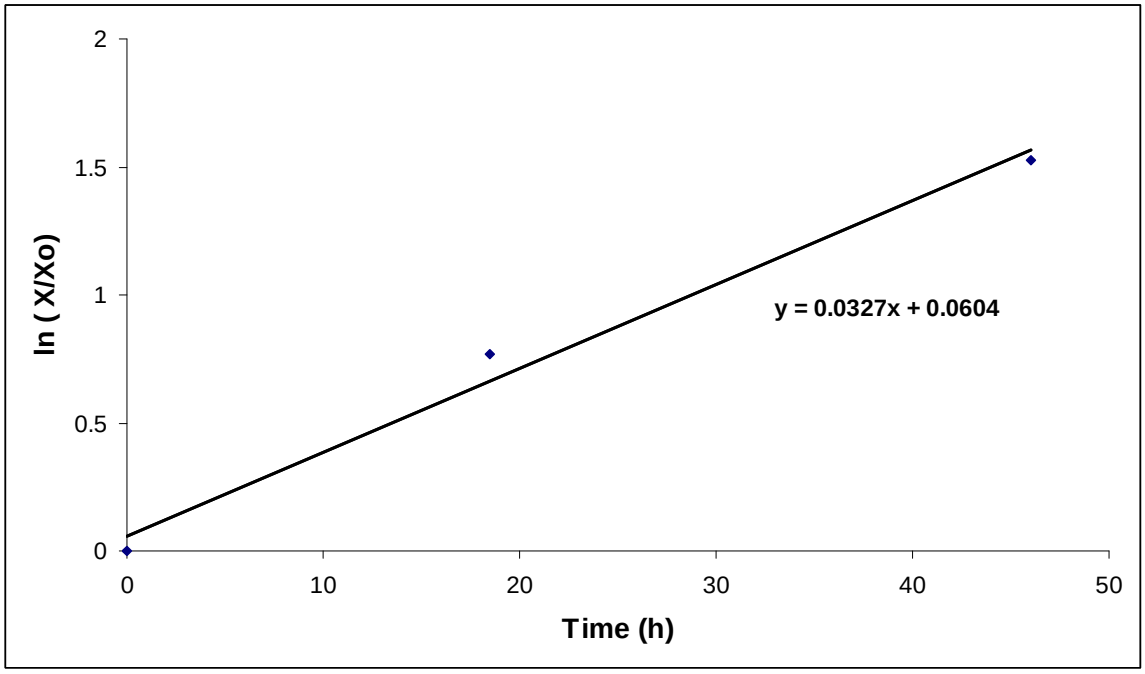

Figure 4.4: Growth curve for control. X - Cell mass at time t, Xo – cell mass at t= 0.

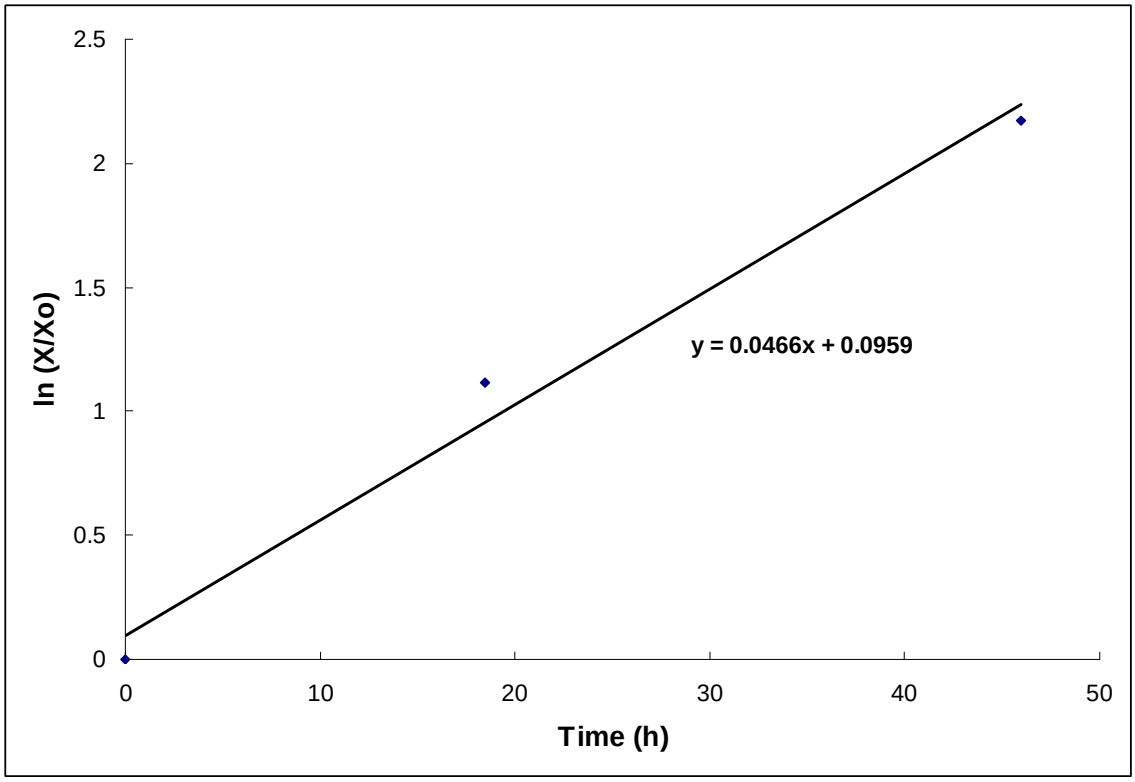

Figure 4.5: Growth curve for cell grown on 0.6% glucose. X - Cell mass at time t, Xo – cell mass at t= 0.

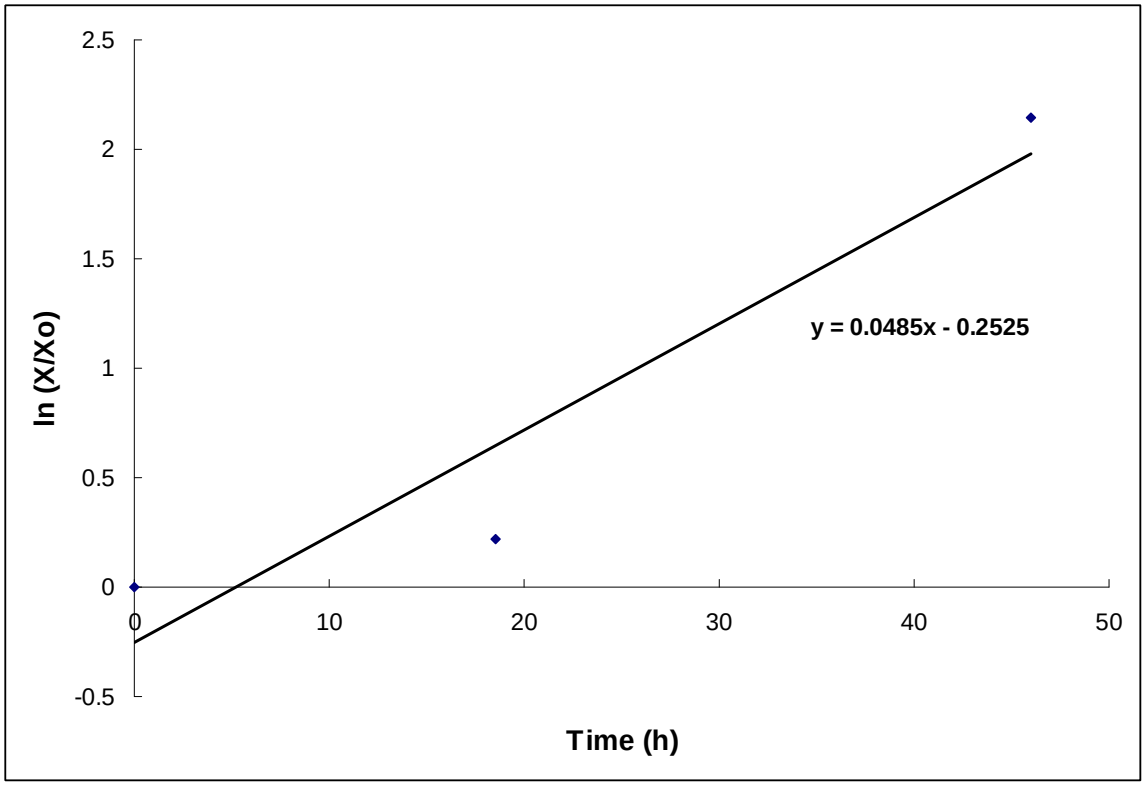

Figure 4.6: Growth curve for cells grown on 1.2% glucose. X - Cell mass at time t, Xo – cell mass at t= 0.

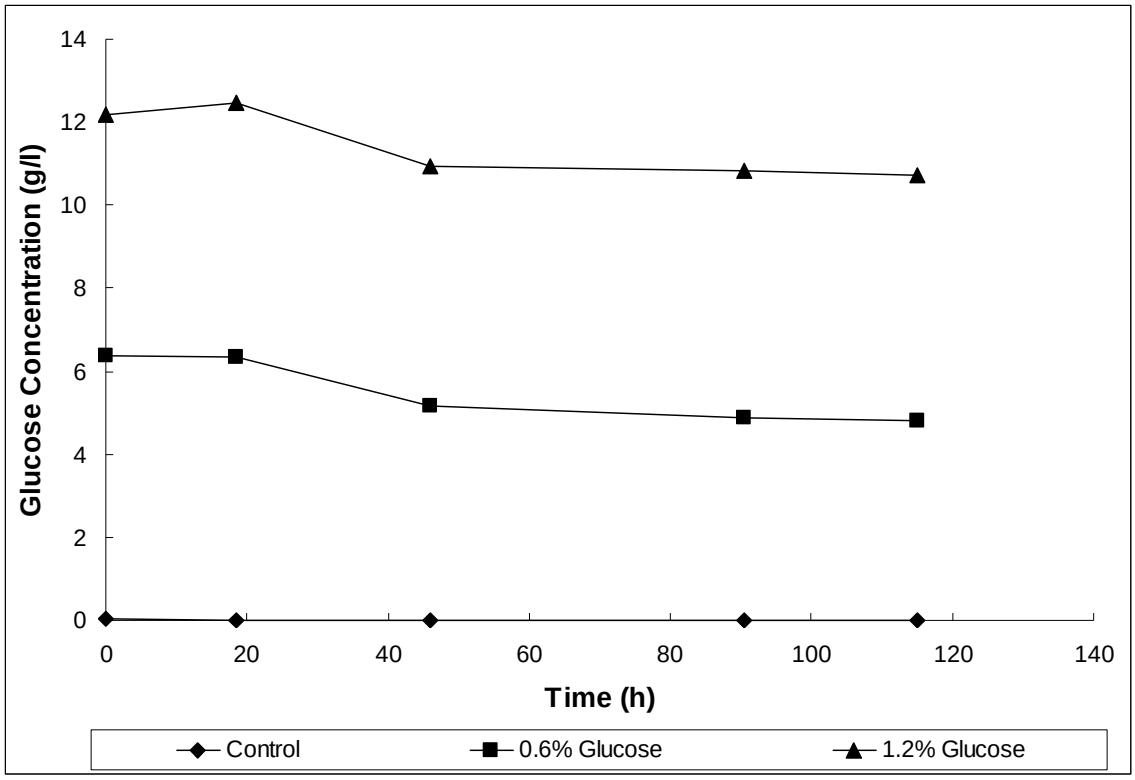

Figure 4.7: Glucose consumption by P11

4.3.2. pH Profile:

As shown in Figures 4.8 and 4.9, the cells exhibited a similar pH pattern before and after cell transfer under all the three conditions. The pH of the medium was initially adjusted to 6 before inoculation. The subsequent decrease in pH was observed with the growth of cells, indicating the production of acetic acid.

4.3.3. Product Profile:

Figures 4.10, 4.11, 4.12 and 4.13 show the product concentration profiles for acetic acid and ethanol before and after cell transfer. The acid profile shown in Figure 4.10 shows the concentration of acetic acid before the cells were transferred. The cells started producing acetic acid by 20h in all cases and a steady increase in the acid concentration could be observed. Moreover, this observation of increase in acid concentration correlates with the increase in cell growth and pH decrease as shown by Figures 4.2 and 4.8. Even though the acetic acid concentration before cell transfer shows a similar pattern under all cases, it can be observed that the cells grown on glucose produced more acetic acid than those grown on syngas. The maximum acetic acid concentrations observed before cell transfer were 2.67g/l in 1.2% glucose, 2.43g/l in 0.6% glucose and 1.93g/l in the control. Figure 4.11 shows the ethanol concentration before the transfer of cells. It can be observed that the cells grown on glucose produced more ethanol than cells grown on syngas. Moreover, an increase in ethanol concentration between 20h and 48h followed by a cessation of ethanol production was observed with the cells grown on glucose while the ethanol concentration increased slowly and consistently in the control. The cells grown on 0.6% and 1.2% glucose started producing ethanol within the first 20h, and they produced a maximum

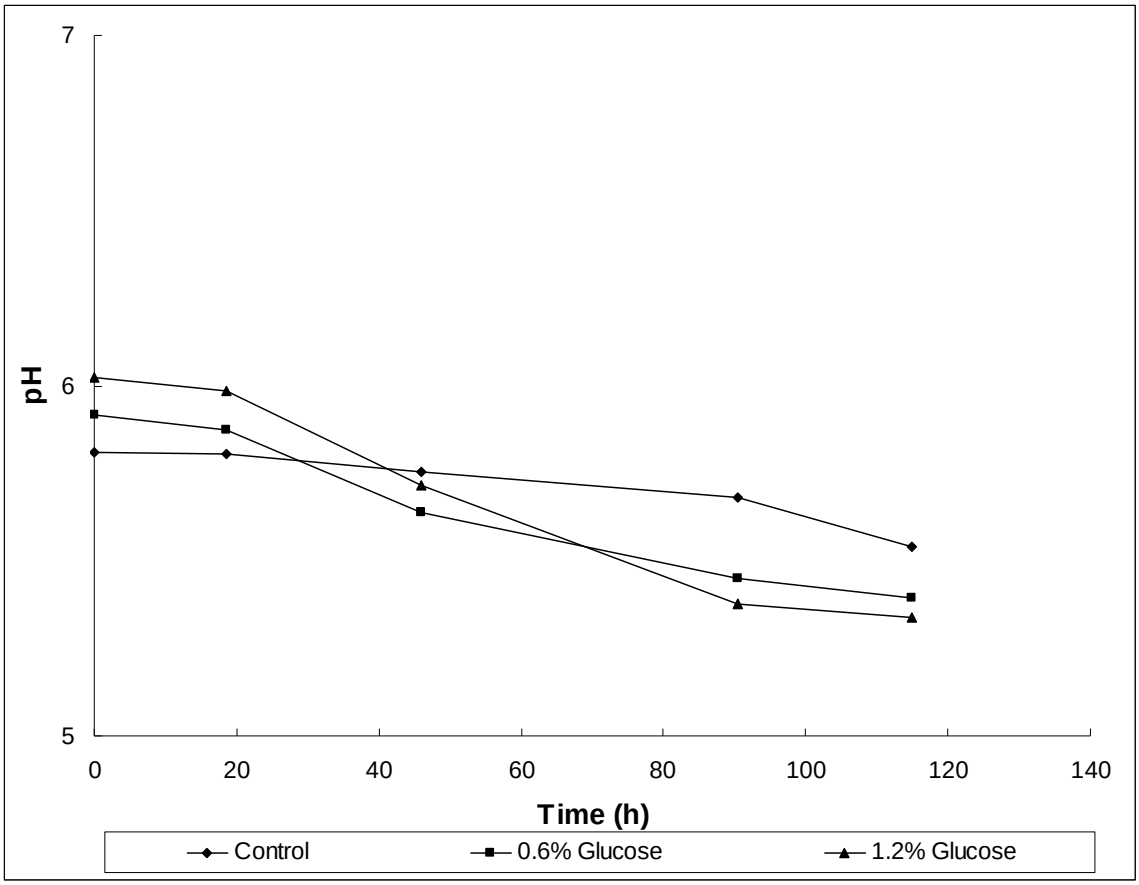

Figure 4.8: pH profile before cell transfer.

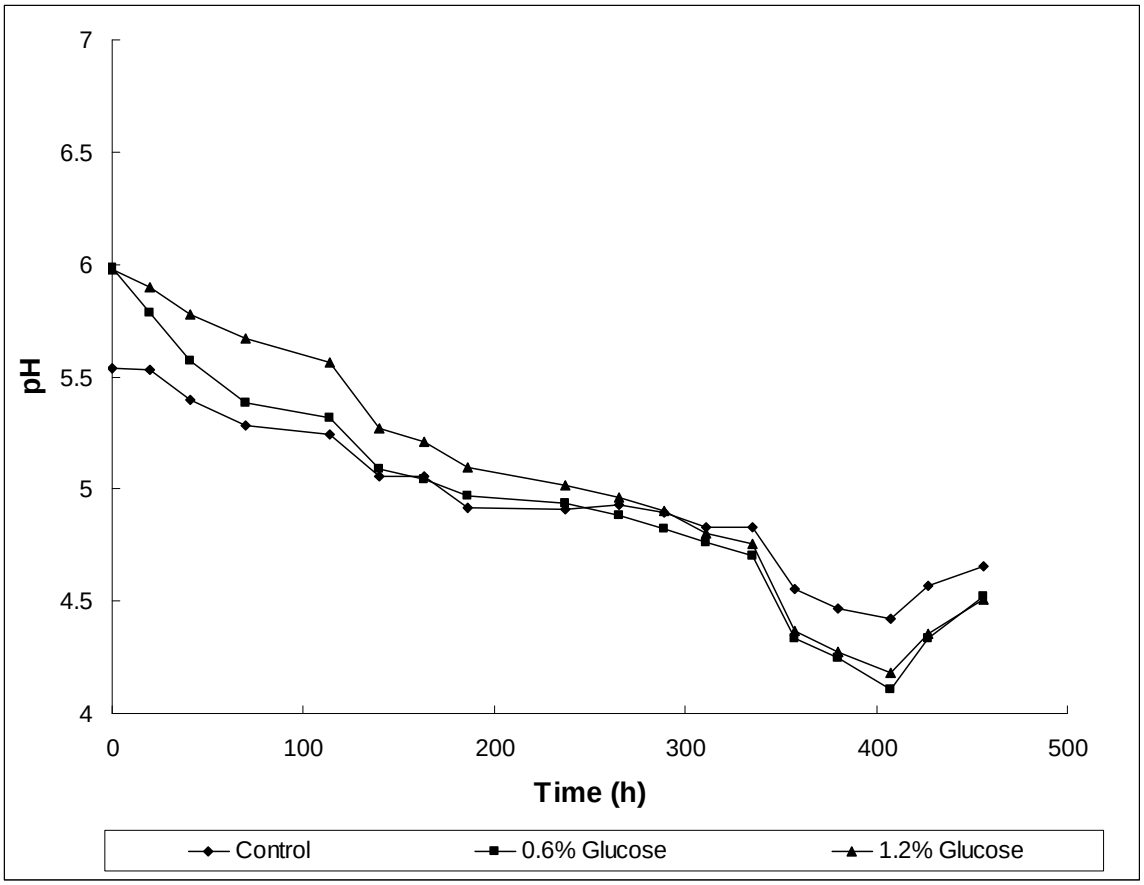

Figure 4.9: pH profile after cell transfer.

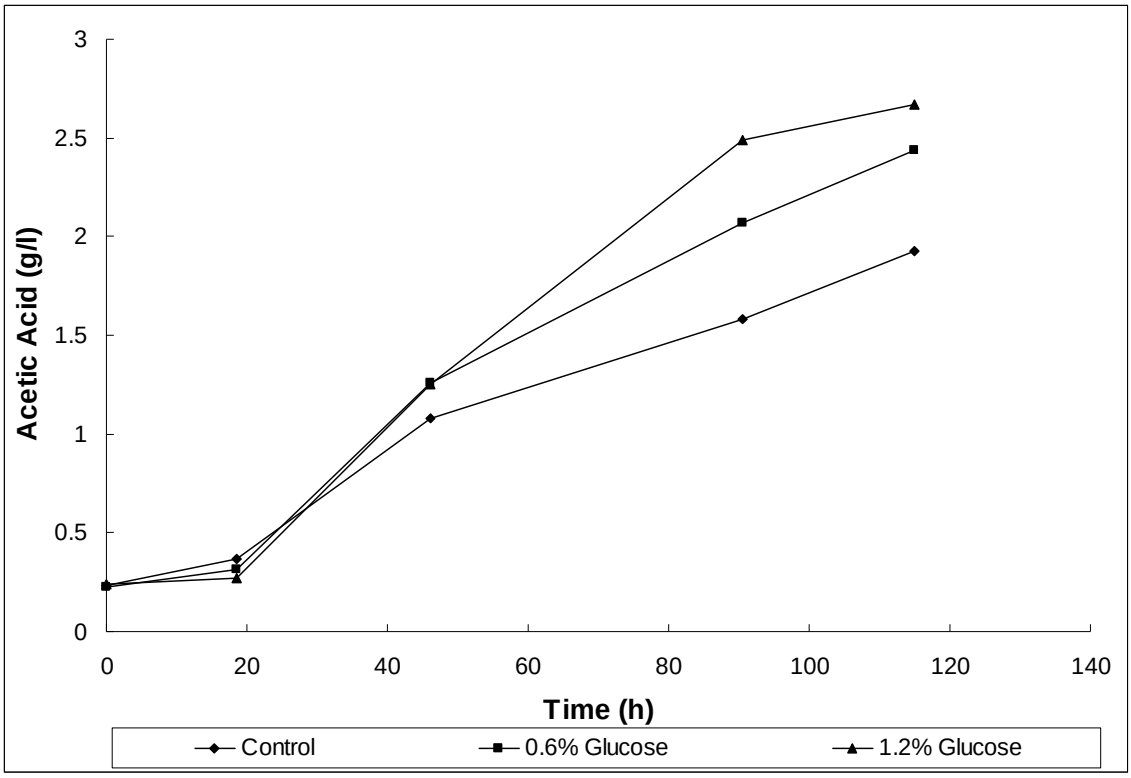

Figure 4.10: Acetic acid concentration before cell transfer.

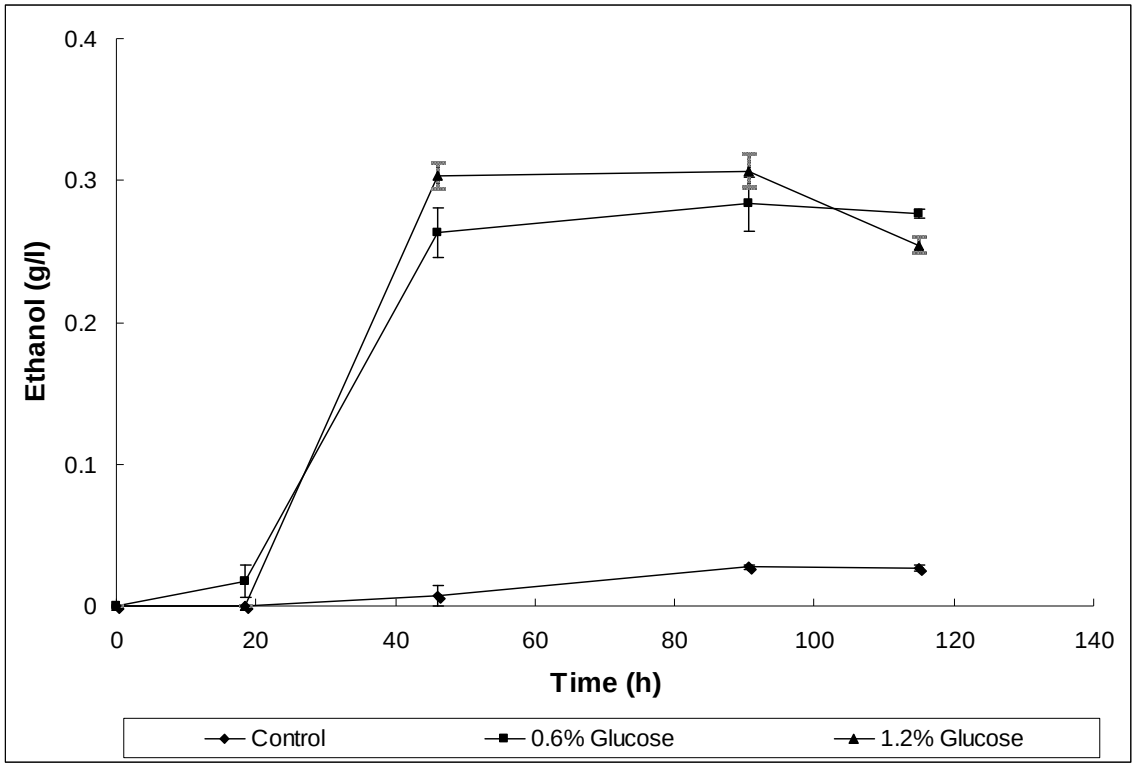

Figure 4.11: Ethanol concentration before cell transfer.

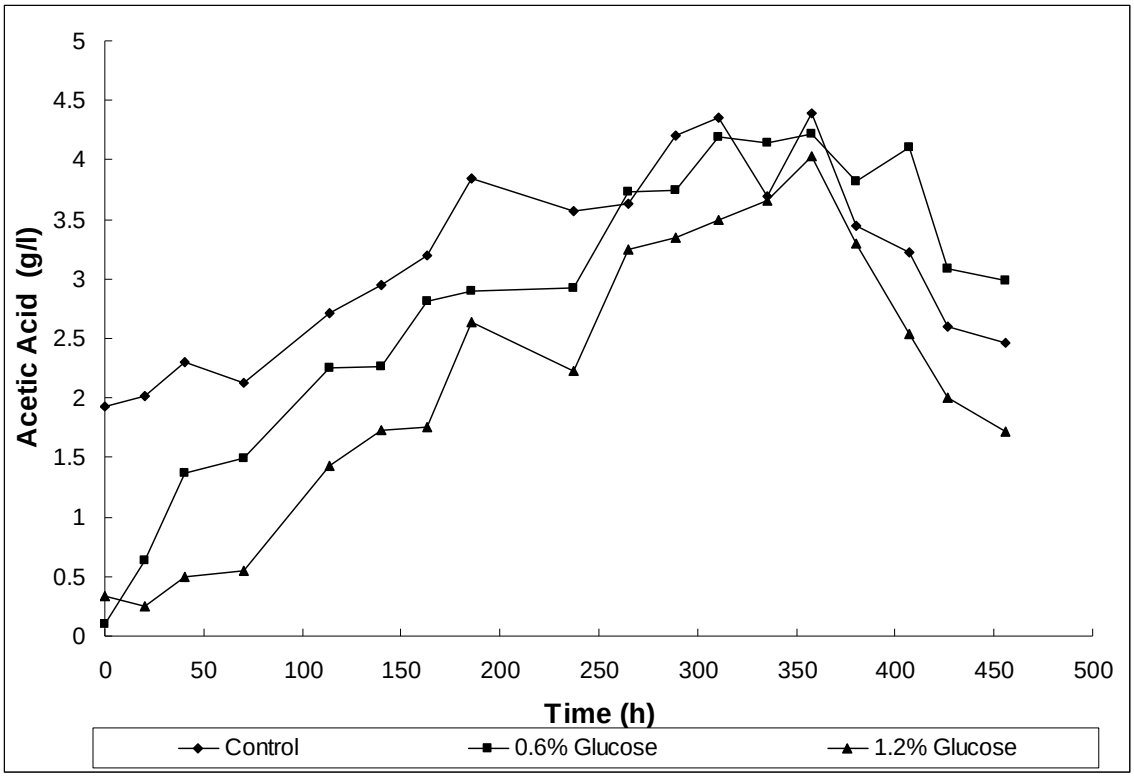

Figure 4.12: Acetic acid concentration after cell transfer.

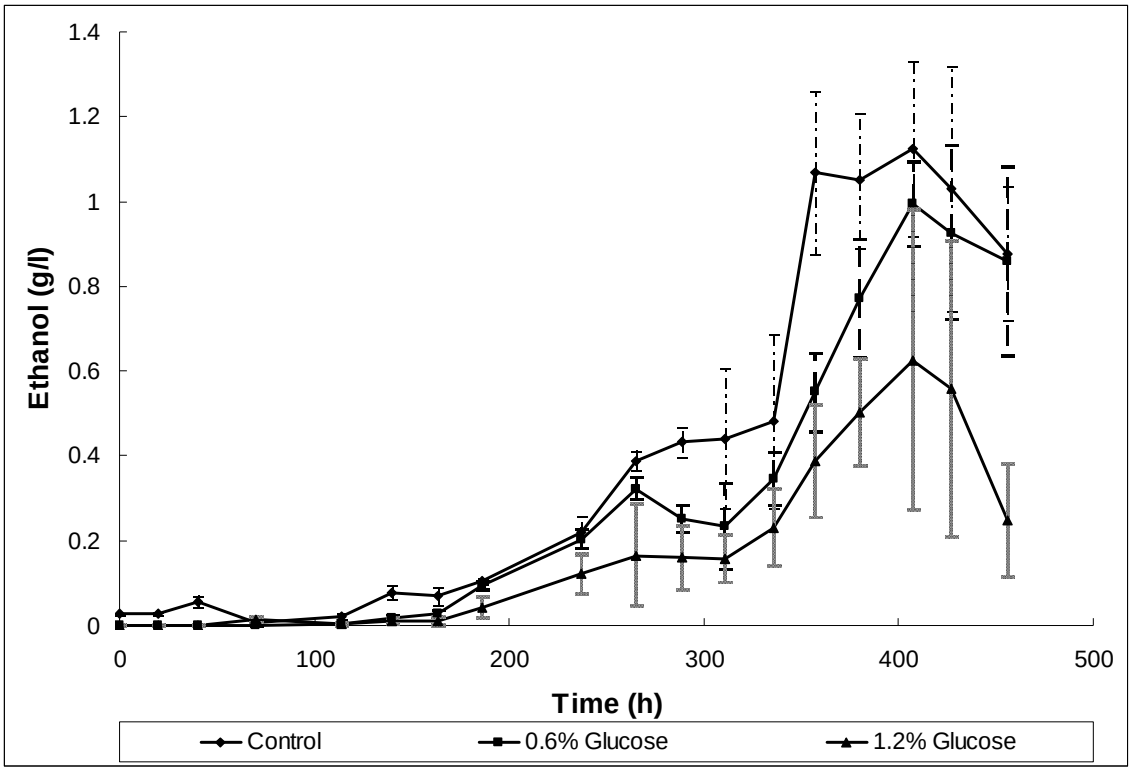

Figure 4.13: Ethanol concentration after cell transfer.

of 0.28g/l and 0.30 g/l at 90h, respectively. The cells grown on syngas produced a maximum of 0.026g/l at 115h. This increase in ethanol concentration observed in the cells grown on glucose could be due to the evolution of hydrogen observed during the production of acetyl-CoA via pyruvate from glucose (Temudo et al. 2007). It is hypothesized that these hydrogen ions, in turn, could have been used by the organism to produce NADH which is associated with ethanol production.

Figure 4.12 shows the acetic acid profile after the transfer of cells. A constant increase in the acetic acid concentration is seen until 186h under all conditions. The acid concentration decreases around 237h for control and the cells grown initially on 1.2% glucose, while the acid concentration levels at 237h for the cells grown initially on 0.6% glucose. This decrease coincides with the start of ethanol production as shown in Figure 4.13, supporting the fact that ethanol is a non-growth related product. Moreover, these organisms follow a biphasic growth pattern, which is indicated by another exponential phase around 186h for the cells grown initially on glucose and at 237h for the cells in the control as observed in Figure 4.3. This exponential phase could be observed for another 60 to 70h before cell growth ceased again. During the second stationary phase, ethanol production increased to a maximum at 407h in all cases which supports the theory that ethanol is non-growth related. The maximum ethanol concentrations were 1.1 g/l in the control, 0.99 g/l for cells grown on 0.6% glucose and to 0.62 g/l for cells grown initially on 1.2% glucose. It can also be seen that acetic acid production decreased at the same time ethanol production increased. This could be due to reduction of acetic acid by P11 to produce ethanol. This previously has been observed by Dr. Ralph Tanner in his lab (Ralph Tanner, Personal communication, 2008). This decrease in ethanol concentration

observed around 427h is due to the cell death, which is evident from the drop in cell and acetic acid concentration. The increase in pH after 427h also indicated cell death.

4.4. CONCLUSION:

The anaerobic conversion of syngas to acetic acid and ethanol was demonstrated under batch conditions using a novel microbial catalyst, P11. Cell mass, ethanol and acetic acid concentrations were determined. The study shows that using glucose as a substrate for inoculum production reduced the lag period and increased cell concentration. However, using glucose to grow an inoculum for syngas fermentation did not increase the maximum ethanol concentration produced after transferring the cells to grow on syngas as their substrate when compared to using syngas to grow inoculum.

CHAPTER 5

EFFECTS OF VARIOUS REDUCING AGENTS ON SYNGAS FERMENTATION BY P11

5.1 INTRODUCTION:

P11 is a strict anaerobe that uses the acetyl-CoA pathway to convert simple substrates such as CO, H_2 and CO_2 to acetic acid. It has been observed that anaerobic metabolism requires a low redox potential for cell growth (Peguin et al. 1994; Rao and Mutharasan 1988). This low redox potential is usually achieved by boiling the growth media, sparging it with an inert gas or adding a reducing agent to remove the oxygen. The addition of reducing agent has been shown to alter the growth rate and electron flow pathways. However, the mechanism by which the reducing agent alters the metabolism is yet to be totally understood. This change in the metabolism directed by the presence of reducing agents, which are artificial electron carriers, results in the change of end products and its distribution. Studies with reducing agents such as sodium sulfide, sodium thioglycolate, ascorbic acid, cysteine and methyl viologen on *Thermoanaerobacter ethanolicus,* a strict anaerobe, showed that an increase in the reducing agents concentrations led to the production of ethanol. However, at high reducing agent concentrations, an inhibitory effect was observed on the growth of the cells (Rao and Mutharasan 1988). A previous study showed that benzyl and methyl viologen shifted metabolism towards alcohol production in *C.acetobutylicum*. This shift was determined

to be the consequence of an increase in NADH concentration in the cells (Rao and Mutharasan 1986). An increase in NADH concentration favors alcohol production (Girbal et al. 1995; Rao and Mutharasan 1987; Rao and Mutharasan 1988). Investigations have further shown that the addition of artificial electron carriers, such as methyl viologen (Peguin et al. 1994) and neutral red (Girbal et al. 1995) to *C.acetobutylicum* can increase alcohol production by altering the NAD/NADH ratio. According to Girbal et al. (1995) the addition of artificial electron carriers replace the ferredoxin in the oxido-reduction reactions catalyzed by the enzymes involved in the distribution of the electron flow. The utilization of these artificial electron carriers deviates electron flow from hydrogen to NADH production by enzymatic regulation of hydrogenase and ferredoxin NAD reductase. As the alcohol forming pathways are dependent on NADH availability, the increased concentration in NADH leads to an increase in alcohol concentration. Studies by Girbal et al. (1995) on *C.acetobutylicum* with neutral red have further proved that the addition of artificial electron carriers promotes the activity of alcohol dehydrogenase (ADH), the enzyme that facilitates the conversion of alcohol from aldehyde, the latter being produced from acetyl-CoA. Thus, a measure of the activity of ADH could suggest the onset of solventogenesis (Girbal et al. 1995). The objective of the chapter is to regulate the metabolism towards solventogenesis by the addition of artificial electron carriers/ reducing agents, specifically benzyl viologen, methyl viologen and neutral red.

5.2. MATERIALS AND METHODS:

5.2.1 Syngas:

Commercial syngas bought from Superior Specialty Gas, Inc (Tulsa, OK) was used throughout the experiment. The composition used was 5% Hydrogen, 15 % carbon dioxide, 20% carbon monoxide and 60 % nitrogen. The above mentioned composition was used as it is equivalent to the composition of syngas obtained through fluidized bed gasifier using switchgrass as substrate (Datar 2003).

5.2.2 Microbial catalyst and culture medium:

P11 was provided by Dr. Ralph Tanner, University of Oklahoma. This bacterium is capable of fermenting gases CO, CO_2, and H_2, and sugars to produce alcohols and acids. The organism was grown under strict anaerobic conditions.

A defined medium containing minerals, trace metals, vitamins and reducing agents was used to prepare the inoculum and cultivate cells. The mineral stock solution contained (per liter) 100g ammonium chloride, 4g calcium chloride, 20g magnesium sulfate, 10g potassium chloride, 10g potassium phosphate monobasic and 80g sodium chloride. The trace metal composition (per liter) was 0.2 g cobalt chloride, 0.8 g ferrous ammonium sulfate, 1g manganese sulfate, 0.2g nickel chloride, 2g nitrilotriacetic acid, 0.02g sodium molybdate, 0.1g sodium selenate, 0.2g sodium tungstate and 1g zinc sulfate. The stock vitamin solution contained 0.005g of p-amino benzoic acid, 0.002g d-biotin, 0.005g pantothenic acid, 0.002g folic acid, 0.01g MESNA, 0.005g nicotinic acid, 0.01g pyridoxine, 0.005g riboflavin, 0.005g thiamine, 0.005g thioctic acid and 0.005g vitamin B-12. The media contained (per liter) 30ml mineral stock solution, 10ml trace metal solution, 10ml vitamin stock solution, 1g yeast extract, 10g morpholinoethanesulfonic

acid (MES), and 10ml of 4% cysteine-sulfide solution. Resazurin solution (0.1%) was added as a redox indicator. MES was added as a biological buffer to prevent excessive fluctuations in the pH during the course of the experiment. The initial pH of the medium was adjusted to 6 before inoculation.

5.2.3 Batch Studies:

Batch experiments were conducted in 250ml serum bottles with 100ml of liquid media to study the individual effect of benzyl viologen, methyl viologen and neutral red on cell growth, pH and product formation. Media was boiled and purged with nitrogen for 2 min to remove oxygen and allowed to cool. One ml of cysteine sulfide (4%) was added to the bottles to scavenge any remaining dissolved oxygen. Bottles were then sterilized in an autoclave (Primus Sterilizer Co. Inc) at 121ºC for 20 min. Sterilized bottles were allowed to cool. Controls were purged with syngas. The bottles were then inoculated with 10ml of inoculum and placed at 37ºC on a shaker (Innova 2100, New Brunswick Scientific, Edison, NJ) at 150 rpm. Reducing agents were filter-sterilized using 0.22 µm disposal filters and were added at 91h when the cells were in their exponential phase of growth. The time of addition of reducing agents was based on previous experience.

The reducing agents in their oxidized form were supplemented at a final concentration of 0.1 mM. All studies were performed in triplicate and one set three controls contained no reducing agents. Cell concentration, pH, acetic acid and ethanol concentrations were measured at regular intervals.

5.2.4 Analytical Procedures:

The optical density (OD), which is proportional to the cell concentration, was determined using a UV-Vis spectrophotometer (Varian Inc., Palo Alto, CA). Cell samples were

collected from the bottles in 2 ml cuvettes. OD was measured at 660nm. Samples with an OD greater than 0.4 units were diluted so that the OD was within the linear range of calibration.

Collected samples were then centrifuged at 10000 rpm for 10 min. The supernatant was collected and frozen for further analysis. Analysis for acetic acid, ethanol, butanol and isopropanol was done using a 6890 gas chromatograph **(**Agilent Technologies, Wilmington, DE**)** fitted with a flame ionization detector and an 8 ft Porpak QS 80/100 column (Alltech, Deerfield, IL). Injector and detector temperature were held at 175°C and 250°C, respectively, and column temperature of 160°C was maintained for 6 min and was increased to 220°C at the rate of 5°C/min. Helium was used as the carrier gas at a flow rate of 29.6 ml/min for 6 min and was ramped to 42 ml/min at the rate of 1 ml/min^2. Methanol was the internal standard. The total analysis time for each sample was 18 min.

5.3 RESULTS AND DISCUSSION:

5.3.1. Cell Growth:

Figure 5.1 shows the cell growth before and after the addition of reducing agents. The cells in all treatments had a similar growth pattern before the addition of the reducing agents. The addition of reducing agents at 91h led to a decrease in cell concentration while the cell concentration continued to increase in the control. This is because the addition of the redox dyes hinders ATP production required for growth by inhibiting acidogensis (Rao et al. 1987).

Cells with methyl viologen and neutral red showed a similar growth pattern after reducing agent addition. The cells move into their stationary phase at 163h (approx) after a decrease in the cell concentration due to the addition of reducing agents. Cells with

neutral red were in stationary phase for 24h and cells with methyl viologen were in stationary phase for 71h before the onset of a secondary growth phase, as seen in Figure 5.1. The controls continued in their exponential phase for 114h and, similar to the bottles with methyl viologen, shifted to a stationary phase at 163h that lasted for 71h. Bottles with benzyl viologen showed the largest decrease in cell concentration. After the addition of benzyl viologen, the cell concentration decreased for 97h and was followed by a stationary phase which lasted for 22h. The cell concentration then begun to decrease again after a very short increase. Controls also showed a similar pattern as the cells with benzyl viologen after their stationary phase.

5.3.2 pH Profile:

Figure 5.2 shows the pH profile. The pH of the medium was initially adjusted to 6 before inoculation. Medium pH was observed to decrease in all bottles until the addition of the reducing agents. The observed decrease in pH was due to the production of acids by the cells during growth. The pH continued to decrease until 209h in control bottles and the bottles containing methyl viologen and neutral red. In the case of benzyl viologen an increase in the pH value was observed at 114h after which the pH decreased. After 209h, a gradual increase in pH was observed in the bottles containing methyl viologen. This increase coincided with the production of ethanol.

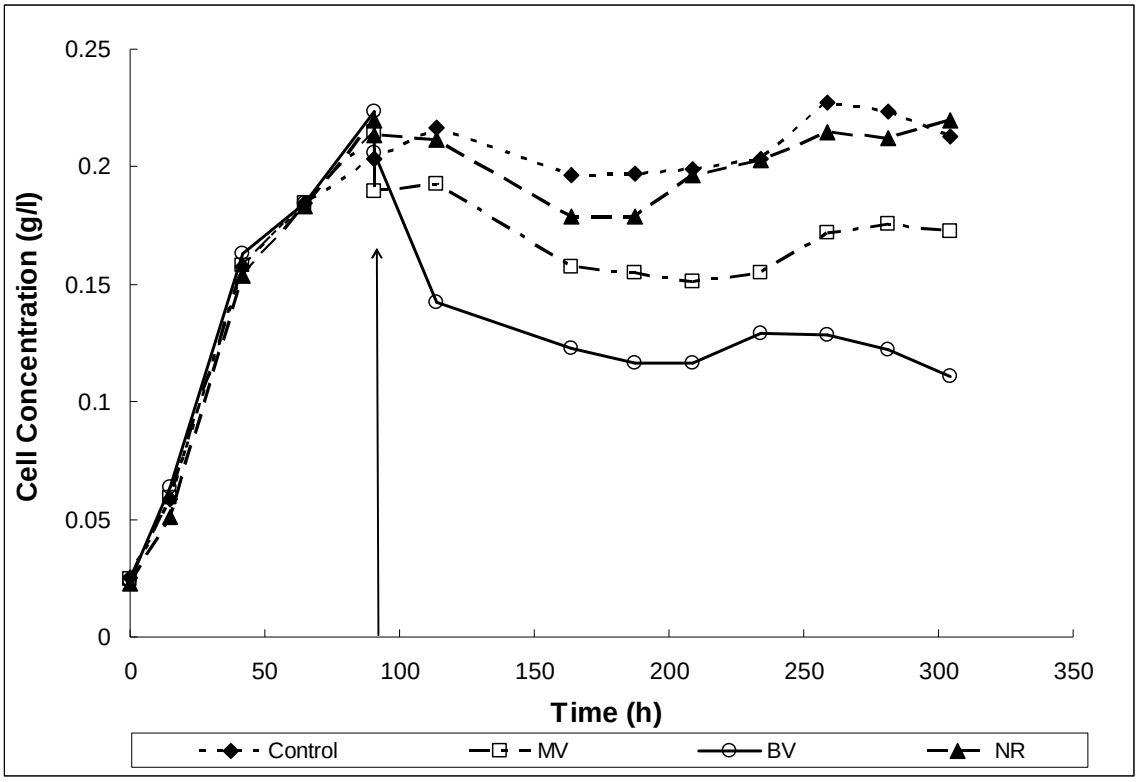

Figure 5.1: Cell growth over time. MV- Methyl viologen, BV- Benzyl viologen and NR – Neutral red. (Arrow indicates the point of addition of reducing agents.)

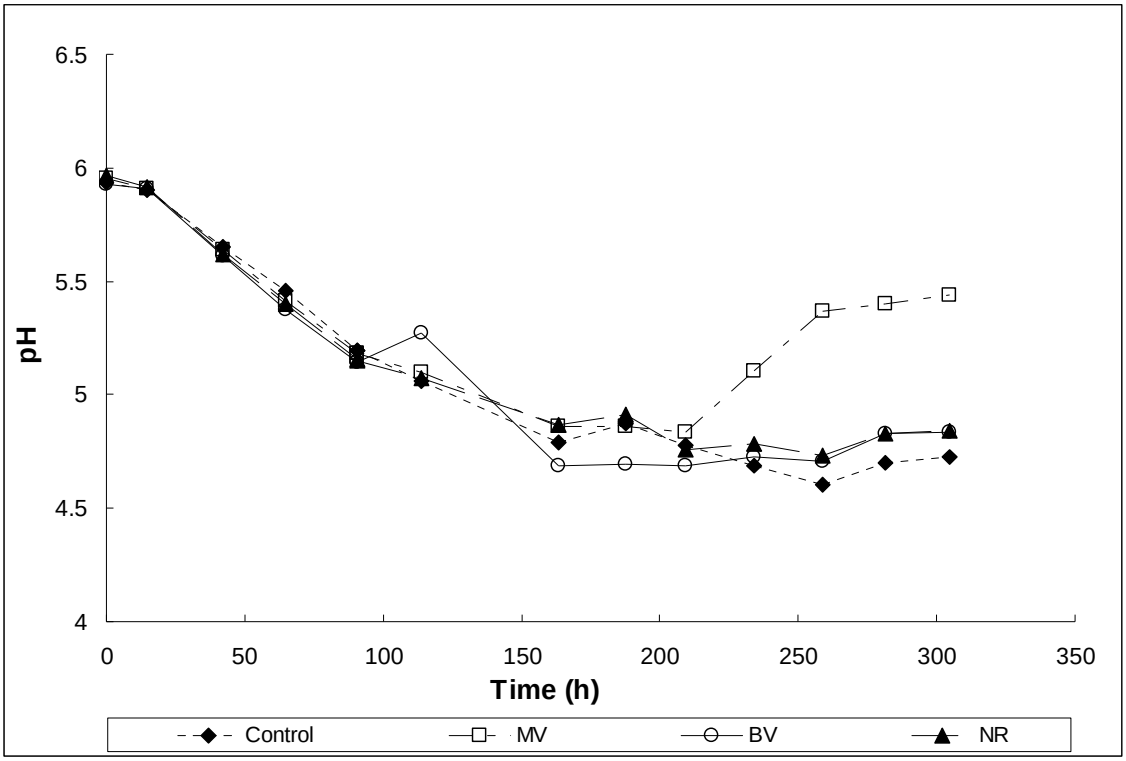

Figure 5.2: pH profile. MV- Methyl viologen, BV- Benzyl viologen and NR – Neutral red.

5.3.3 Product Profile:

Figures 5.3 and 5.4 show the concentration of acetic acid and ethanol produced, respectively. No isopropanol or butanol was detected in any of the treatments.

In Figure 5.3, a constant increase in the acetic acid concentration was seen in all the bottles prior to the addition of the reducing agents. This coincides with the decrease in pH in Figure 5.2. A decrease in acetic acid concentration was observed after the addition of the reducing agents due to dilution of media by the reducing agent solution.

Figure 5.4 shows the concentration of ethanol produced. It can be seen that the bottles containing benzyl viologen did not produce ethanol, while the onset of solvent production in all the other treatments were observed at the same time (209h). This coincides with the increase in pH observed at 234h in Figure 5.2. The highest concentration of ethanol produced was 1.3g/l by the cells to which methyl viologen was added. Cells with neutral red produced a maximum of 0.62g/l while the cells with no added reducing agents (control) produced 0.51g/l. The higher concentration of solvent observed in the presence of reducing agents such as neutral red and methyl viologen was hypothesized to be the consequence of the offset in NADH/NAD+ ratio brought out by these agents. The reducing agents which are actually artificial electron carriers, are oxidized when added. The electrons donated by the agents lead to an increase in the NADH concentration, which ultimately leads to the promotion of the solventogenesis branch of the pathways to maintain the NADH/NAD+ ratio (Hipolito et al. 2008). The observed difference in ethanol concentration indicates the manipulation of the metabolic pathway by the addition of reducing agents. Furthermore, it can be seen that the reducing agents methyl viologen and neutral red promoted ethanol production earlier than the control, i.e. cells

started producing ethanol at 209h in the presence of methyl viologen and at 234h with neutral red while the control produced ethanol after 234h. This indicates that the addition of reducing agents advanced the onset of solventogenesis as seen in previous studies conducted by Hipolito et. al (2008), Peguin et al. (1994), Rao and Mutharasan (1986). Figure 5.5 shows the effect of reducing agents on ethanol yield expressed as g ethanol per cell mass. Treatments containing methyl viologen showed the highest ethanol yield (7.5g/g) while benzyl viologen had the lowest (0g/g). Bottles containing neutral red and controls had 2.8g/g and 2.4g/g of ethanol per cell mass, respectively. The change in ethanol concentration was also evaluated using an analysis of variance (ANOVA) test with time included as a factor (general linear model) and Dunnett's test performed using SAS® (SAS Institute, Cary, NC). The statistical analysis showed that there was no difference between the different treatments at 234h and 259h. However, addition of methyl viologen had a positive effect compared to the control at 281h and 305h while the effect of neutral red was not different than the control. The statistical analysis was done after removing a methyl viologen sample at 305h which was an outlier.

Similar studies conducted by Ahmed (2006) on *C.carboxidivorans* with 0.1mM neutral red in batch reactors increased ethanol yield from 0.05g/g cells to 0.2 g/g cells. Experiments conducted by Rao and Mutharasan (1986) with methyl viologen in a continuous reactor at a concentration of 6mg/L increased the ethanol production by seven fold in *C.acetobutylicum*.

Regarding the effect of benzyl viologen, an optimum concentration that is lower than 0.1mM might be required to boost the alcohol production. Concentrations of 0.2mM, 0.3mM, 0.4mM and 0.5mM proved to be inhibitory to cell growth. Results of these

studies on cell growth are shown in Figure 5.6 and 5.7. Higher concentrations of reducing agents are known to have an inhibitory effect on the cells.

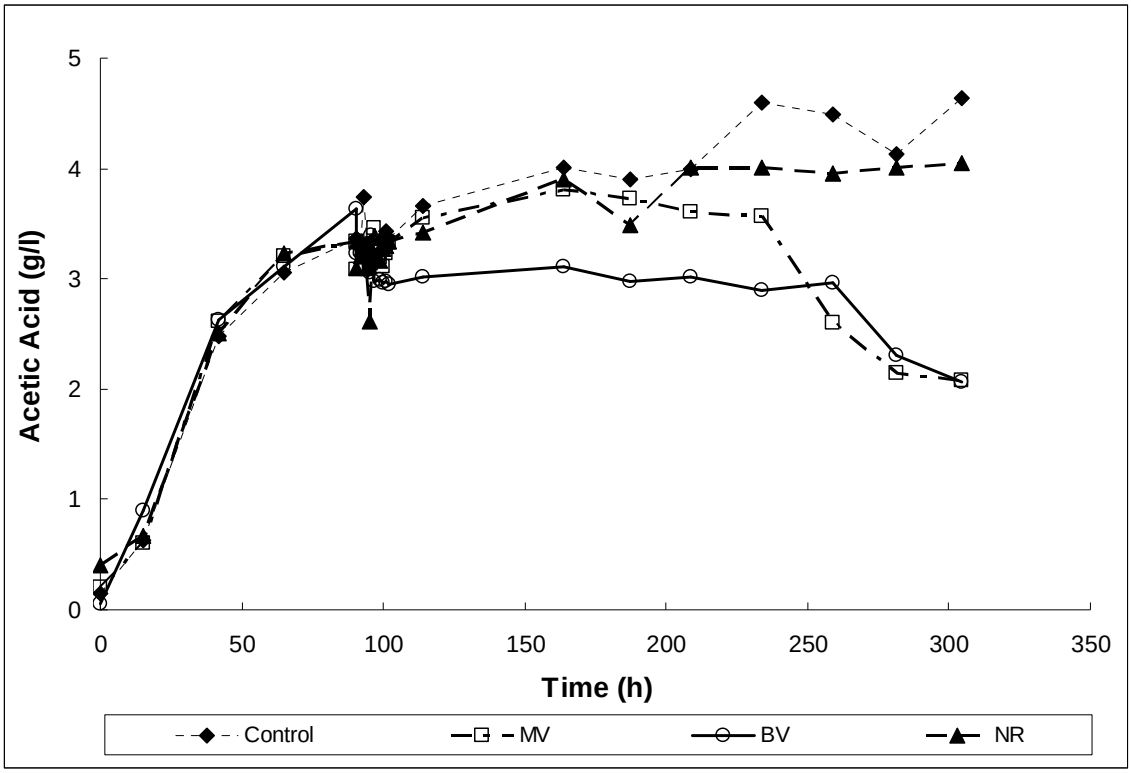

Figure 5.3: Acetic acid concentration (g/l). MV- Methyl viologen, BV- Benzyl viologen and NR – Neutral red.

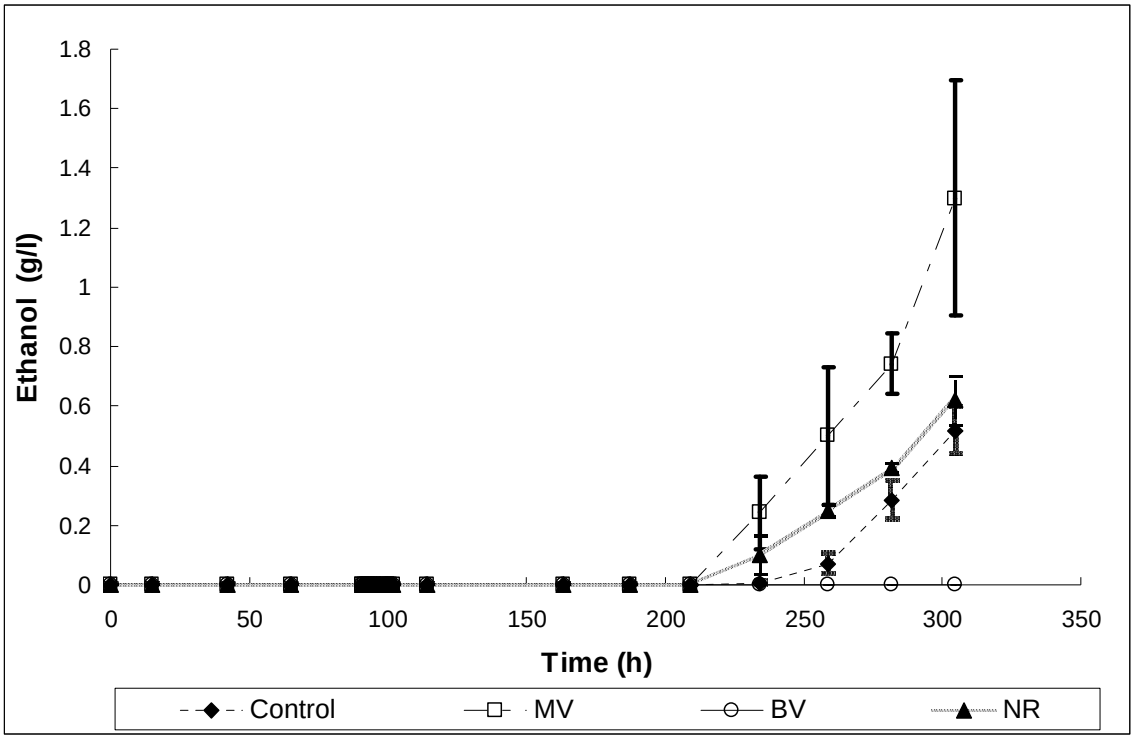

Figure 5.4: Ethanol concentration. Error bars represent ± 1 standard error (n =3).

MV- Methyl viologen, BV- Benzyl viologen and NR – Neutral red.

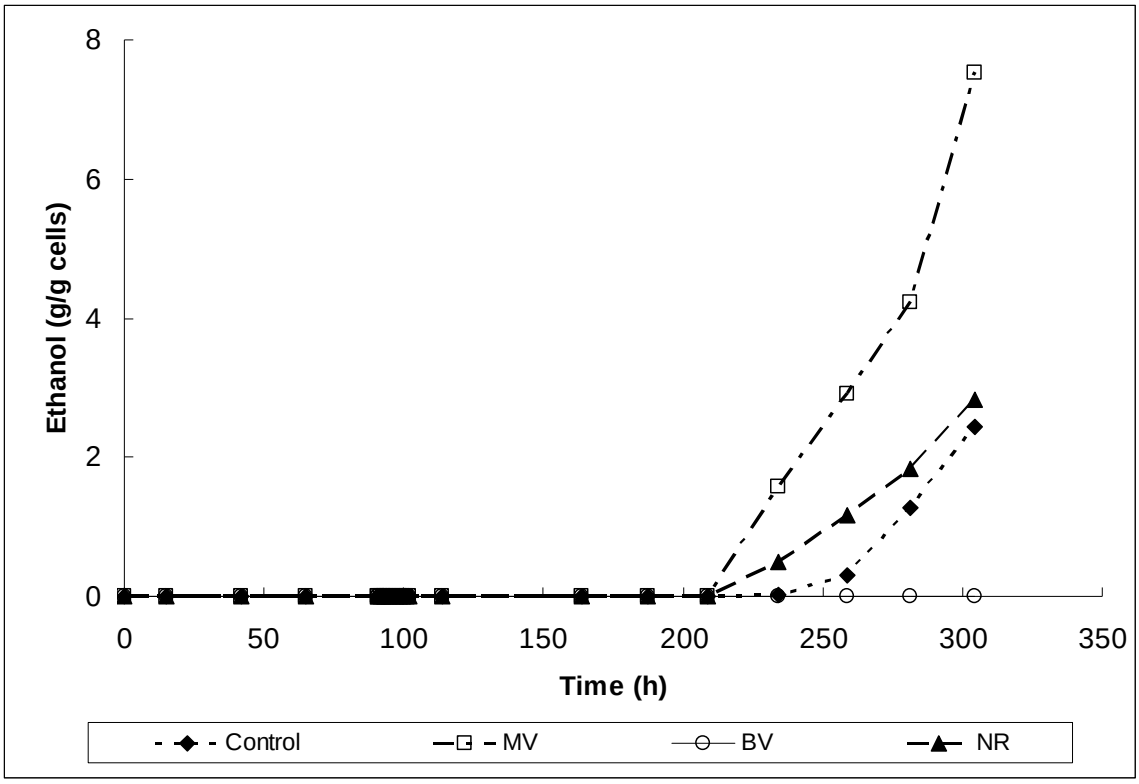

Figure 5.5: Effect of various reducing agents on ethanol per cell mass. MV- Methyl viologen, BV- Benzyl viologen and NR – Neutral red.

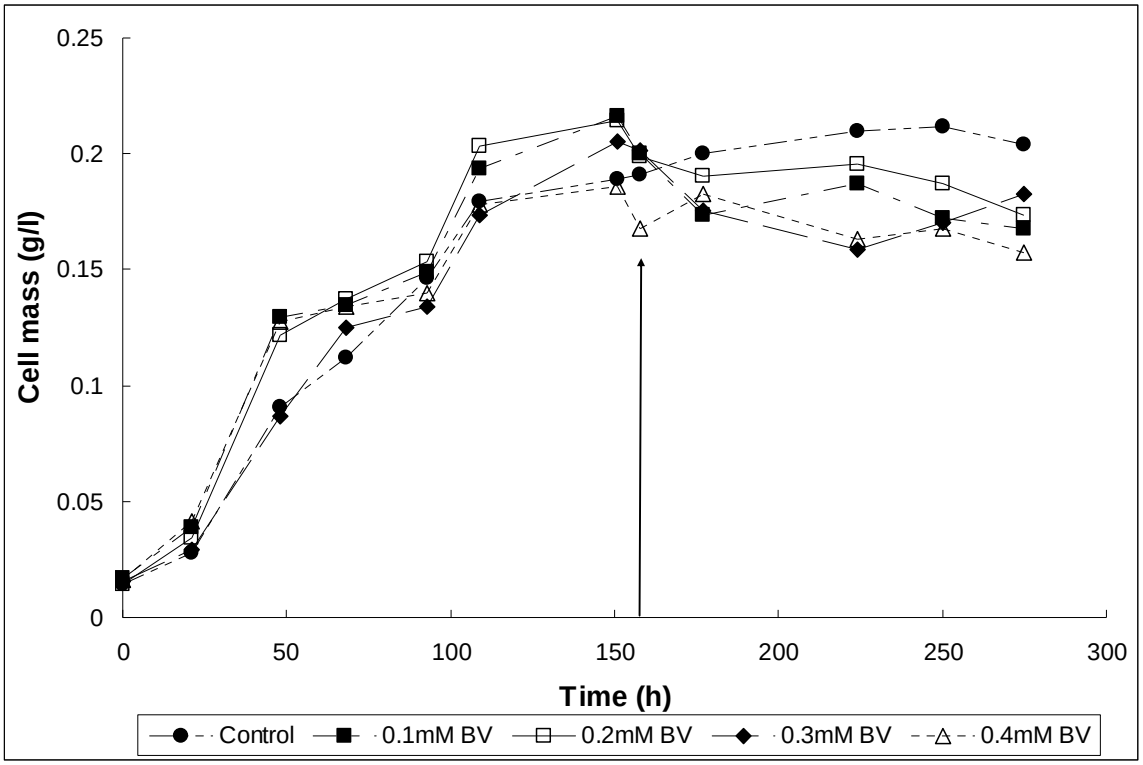

Figure 5.6: Effect of benzyl viologen (BV) of various concentrations on cell growth (Arrow indicates the point of addition of benzyl viologen).

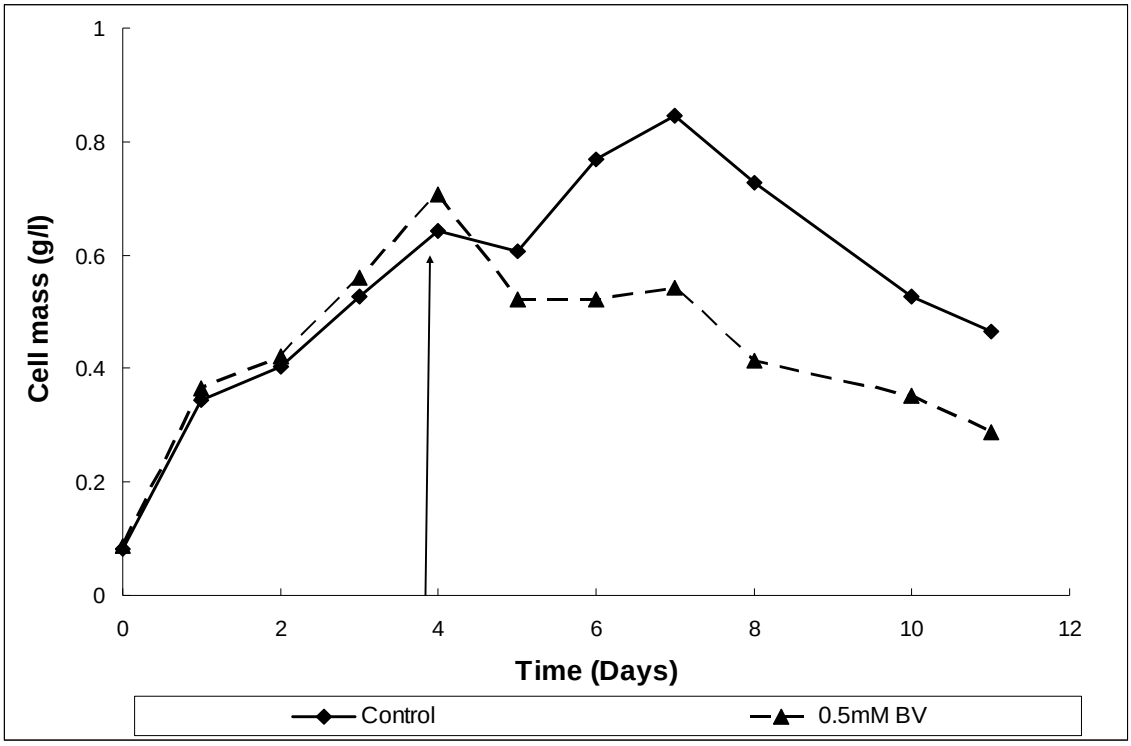

Figure 5.7: Effect of 0.5mM of benzyl viologen (BV) on cell growth (Arrow indicates the point of addition of benzyl viologen).

5.4 Conclusion:

Studies were conducted to investigate the effect of reducing agents/ artificial electron carriers on syngas fermentation using P11. Cell growth and product concentration were determined. The study shows that the addition of 0.1mM of methyl viologen produced maximum ethanol concentration of 1.3g/l while cells with neutral red and control produced 0.62g/l and 0.51g/l of ethanol. The addition of 0.1mM of benzyl viologen prevented solventogenesis. Furthermore, methyl viologen also decreased the amount of acetic acid produced.

CHAPTER 6

FUTURE WORK

Reduction of lag period and increase of ethanol yield could be done by various means. Some suggestions for the future are:

- The effect of other sugars such as fructose, galactose, arabinose, etc. as the initial substrate could be studied.
- The optimum concentration of these sugars, including glucose, could be estimated to reduce the lag period.
- The technique of cell immobilization could be used to increase the cell mass, which might also lead to increased ethanol concentration.

The metabolic regulation study could be broadened. Some suggestions are:

- Determination of an optimum concentration of methyl viologen and neutral red could increase the ethanol yield.
- The yield of ethanol might increase by down regulating the enzymes that are responsible for the acidogenesis phase. The enzymes involved in this phase are: phosphotransacetylase and acetate kinase. Phosphotransacetylase is inhibited by the compounds such as 2,3-butanedione and desulfo- CoA (Iyer and Ferry 2001). Acetate kinase, the enzyme that catalyzes the reaction in which acetate is produced from acetyl phosphate, is inhibited by potassium chloride

(Lin et al. 1998), mercuric chloride and para- hydroxymercuricbenzoate (Winzer et al. 1997). Inhibition or the down regulation of these enzymes might lead to an increase in ethanol production.

- Respiratory inhibitors, such as sodium azide and dinitrophenol, have increased the ethanol yield in yeasts, when used at very low concentrations (Singh et al. 1991). The effects of these compounds could be studied using P11.

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APPENDIX

Program for LSD Test in Chapter 5

```
options ls=74 ps=60;
data P11234;
infile "h:Anusha Thesis\234hethanol.csv" dlm=",";
input ea$ eth234 @@;
cards;
run;
data P11259;
infile "h:Anusha Thesis\259hethanol.csv" dlm=",";
input ea$ eth259 @@;
cards;
run;
data P11281;
infile "h:Anusha Thesis\281hethanol.csv" dlm=",";
input ea$ eth281 @@;
cards;
run;
data P11305;
infile "h:Anusha Thesis\305hethanola.csv" dlm=",";
input ea$ eth305 @@;
cards;
run;
proc glm data=P11234; class ea;
model eth234 = ea;
means ea/lsd;
```

```
run;
proc glm data=P11259; class ea;
model eth259 = ea;
means ea/lsd;
run;
run;proc glm data=P11281; class ea;
model eth281 = ea;
means ea/lsd;
run;
run;proc glm data=P11305; class ea;
model eth305 = ea;
means ea/lsd;
run;
```

Output from SAS Release 9.1

The GLM Procedure

Class Level Information

Class	Levels	Values
ea	4	BV C MV NR

Number of Observations Read	12
Number of Observations Used	12

The GLM Procedure

Dependent Variable: eth234

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.11552492	0.03850831	2.68	0.1176
Error	8	0.11486733	0.01435842		
Corrected Total	11	0.23039225			

R-Square	Coeff Var	Root MSE	eth234 Mean
0.501427	137.3371	0.119827	0.087250

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ea	3	0.11552492	0.03850831	2.68	0.1176

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ea	3	0.11552492	0.03850831	2.68	0.1176

The GLM Procedure

t Tests (LSD) for eth234

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	0.014358
Critical Value of t	2.30600
Least Significant Difference	0.2256

Means with the same letter are not significantly different.

t Grouping	Mean	N	ea
A	0.24233	3	MV
A			
B A	0.10100	3	NR
B			
B	0.00567	3	C
B			
B	0.00000	3	BV

The GLM Procedure

Class Level Information

Class	Levels	Values
ea	4	BV C MV NR

Number of Observations Read	12
Number of Observations Used	12

The GLM Procedure

Dependent Variable: eth259

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.45126692	0.15042231	3.63	0.0641
Error	8	0.33113600	0.04139200		
Corrected Total	11	0.78240292			

R-Square	Coeff Var	Root MSE	eth259 Mean
0.576770	99.44615	0.203450	0.204583

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ea	3	0.45126692	0.15042231	3.63	0.0641

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ea	3	0.45126692	0.15042231	3.63	0.0641

The GLM Procedure

t Tests (LSD) for eth259

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	0.041392
Critical Value of t	2.30600
Least Significant Difference	0.3831

Means with the same letter are not significantly different.

t Grouping	Mean	N	ea
A	0.5013	3	MV
A			
B A	0.2487	3	NR
B			
B	0.0683	3	C
B			
B	0.0000	3	BV

The GLM Procedure

Class Level Information

Class	Levels	Values
ea	4	BV C MV NR

Number of Observations Read	12
Number of Observations Used	12

The GLM Procedure

Dependent Variable: eth281

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.84913867	0.28304622	25.56	0.0002
Error	8	0.08860200	0.01107525		
Corrected Total	11	0.93774067			

R-Square	Coeff Var	Root MSE	eth281 Mean
0.905515	29.67265	0.105239	0.354667

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ea	3	0.84913867	0.28304622	25.56	0.0002

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ea	3	0.84913867	0.28304622	25.56	0.0002

The GLM Procedure

t Tests (LSD) for eth281

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	0.011075
Critical Value of t	2.30600
Least Significant Difference	0.1981

Means with the same letter are not significantly different.

t Grouping	Mean	N	ea
A	0.74333	3	MV
B	0.39067	3	NR
B			
B	0.28467	3	C
C	0.00000	3	BV

The GLM Procedure

Class Level Information

Class	Levels	Values
ea	4	BV C MV NR

Number of Observations Read	11
Number of Observations Used	11

The GLM Procedure

Dependent Variable: eth305

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	1.11676364	0.37225455	32.70	0.0002
Error	7	0.07969000	0.01138429		
Corrected Total	10	1.19645364			

R-Square	Coeff Var	Root MSE	eth305 Mean
0.933395	22.47116	0.106697	0.474818

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ea	3	1.11676364	0.37225455	32.70	0.0002

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ea	3	1.11676364	0.37225455	32.70	0.0002

The GLM Procedure

t Tests (LSD) for eth305

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	7
Error Mean Square	0.011384
Critical Value of t	2.36462

Comparisons significant at the 0.05 level are indicated by ***.

ea Comparison	Difference Between Means	95% Confidence Limits		
MV - NR	0.28600	0.05568	0.51632	***
MV - C	0.38900	0.15868	0.61932	***
MV - BV	0.90600	0.67568	1.13632	***
NR - MV	-0.28600	-0.51632	-0.05568	***
NR - C	0.10300	-0.10300	0.30900	
NR - BV	0.62000	0.41400	0.82600	***
C - MV	-0.38900	-0.61932	-0.15868	***
C - NR	-0.10300	-0.30900	0.10300	
C - BV	0.51700	0.31100	0.72300	***
BV - MV	-0.90600	-1.13632	-0.67568	***
BV - NR	-0.62000	-0.82600	-0.41400	***
BV - C	-0.51700	-0.72300	-0.31100	***

VITA

ANUSHADEVI PANNEERSELVAM

Candidate for the degree of

Master of Science

Thesis: **EFFECT OF GLUCOSE AND REDUCING AGENTS ON SYNGAS FERMENTATION BY *CLOSTRIDIA* SPECIES P11**.

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Professional Membership: American Society of Agricultural and Biological Engineers.

Name: Anushadevi Panneerselvam Date of Degree: May 2009

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: EFFECT OF GLUCOSE AND REDUCING AGENTS ON SYNGAS FERMENTATION BY *CLOSTRIDIA* SPECIES P11

Pages of Study: 82 Candidate for the degree of Master of Science

Major Field: Biosystems Engineering.

Scope and Method of Study: The purpose of this study was to determine the effect of glucose and reducing agents on syngas fermentation using P11. The first objective of the study is the effect of glucose, this study was conducted to overcome the lag period and to increase cell concentration to increase ethanol yield. Second objective of the study is the effect of various reducing agents such as methyl viologen, benzyl viologen and neutral red on fermentation using P11. This study was based on the hypothesis that these reducing agents are capable of altering the metabolism towards solventogenesis by disrupting the NADH/NAD ratio.

Findings and Conclusion: The glucose study shows that the initial growth of cells on glucose helps to reduce the lag period and to increase the cell concentration. However, the use of glucose to grow an inoculum for syngas fermentation did not increase the maximum ethanol concentration produced when compared to using syngas to grow inoculum.

The study with reducing agents shows that the addition of 0.1mM of methyl viologen produced maximum ethanol concentration of 1.3g/l while neutral red produced 0.6g/l of ethanol and control produced 0.5g/l of ethanol.0.1mM of benzyl viologen did not have any produce ethanol. Furthermore, methyl viologen also decreased the amount of acetic acid produced. Optimum concentrations of reducing agents are required to up regulate the solventogenesis in P11.

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